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(54) **Title:** CHEMICAL REAGENTS AND METHODS FOR DETECTION AND QUANTIFICATION OF PROTEINS IN COMPLEX MIXTURES
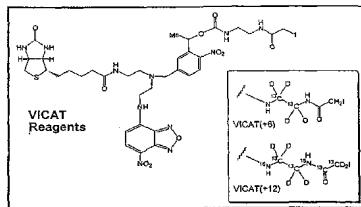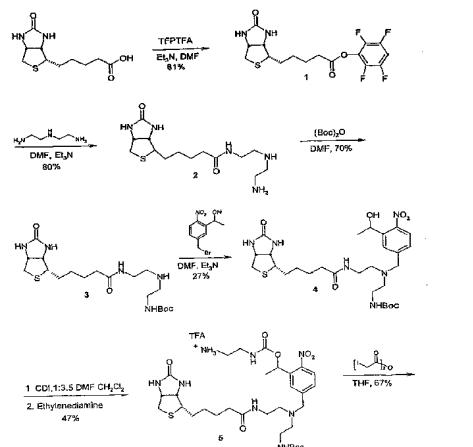
(57) **Abstract:** The invention provides a reagent comprising an affinity tag, a detectable moiety, a linker, an isotope tag and a reactive group. The invention also provides methods of using a reagent of the invention. The methods can be used to label a polypeptide in a sample by contacting a sample with a reagent of the invention under conditions allowing the reactive group to bind one or more polypeptides in the sample. The invention additionally provides methods of isolating, identifying and quantifying a polypeptide in a sample. The invention further provides methods of diagnosing a disease using a reagent of the invention.

CHEMICAL REAGENTS AND METHODS FOR DETECTION AND QUANTIFICATION OF PROTEINS IN COMPLEX MIXTURES

BACKGROUND OF THE INVENTION

The invention is related to the field of analytical biochemistry, and more specifically to the field of quantitative proteomics.

Large scale sequencing of genomic DNA, cDNA and EST's (expressed sequence tags) has identified large numbers of genes. For some selected species, the complete genome sequences have been determined and, for others, EST sequence databases are assumed to contain sequence tags for the majority of genes for that species. For the species having complete genome sequences or substantial EST sequences available, essentially all the genes have been determined. Other genomic technologies including serial analysis of expressed genes (SAGE) and differential cDNA or oligonucleotide array analysis are capable of determining quantitative expression profiles of the expressed genes at the mRNA level in specific cells or tissues. Furthermore, comparative genomic analyses have identified genes that, if defective or aberrantly expressed, are the cause of genetic or somatic diseases.

From genomic analysis or the analysis of the expressed mRNA transcripts neither the quantity nor the structure, activity and state of modification of the translated protein products can be predicted. Recently

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developed mass spectrometric techniques allow the rapid
identification of expressed proteins by the correlation
of mass spectral data that are idiotypic for the
sequence of a specific protein with the sequences
contained in sequence databases.

A number of approaches have been used to
address the needs of proteomics analysis, including the
use of reagents that allow relative quantitation of
proteins in a sample. However, none of the previously
described methods can be used to determine the absolute
quantity of the proteins in a sample. Furthermore,
these methods do not allow the analysis to be focused
on selected proteins such as those that are involved in
specific diseases or those that are predicted or
expected to be present based on circumstantial data or
considerations.

Thus, there exists a need for reagents
suitable for absolute quantitative analysis of protein
samples as well as reagents suitable for analysis of
selected proteins, which can be used as diagnostic or
prognostic markers. The present invention satisfies
these needs, and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides a reagent comprising
an affinity tag, a detectable moiety, a linker, an
isotope tag and a reactive group, referred to herein as
a VICAT reagent. The invention also provides methods
of using a reagent of the invention. The methods can
be used to label a polypeptide in a sample by
contacting a sample with a reagent of the invention
under conditions allowing the reactive group to bind to
one or more polypeptides in the sample. The invention
additionally provides methods of isolating, identifying

and quantifying a polypeptide in a sample. The
invention further provides methods of diagnosing a
disease using a reagent of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the structure of a VICAT
reagent having 7-nitrobenz-2-oxa-1,3-diazole (NBD) as
the detectable moiety. Figure 1B shows a detailed
reaction scheme for the synthesis of a VICAT reagent
having NBD as the detectable moiety. The structures of
intermediate compounds 1-7 and final product, compound
8 (VICAT having NBD as detectable moiety), are shown.

Figure 2 shows the structure of a VICAT
reagent having tetramethyl-rhodamine as the detectable
moiety.

Figure 3 shows photocleavage of a VICAT
reagent-peptide conjugate. A portion of a VICAT
reagent depicted in Figure 2 is shown conjugated to a
peptide. The VICAT-peptide conjugate undergoes
photocleavage to generate a peptide intermediate. The
peptide intermediate spontaneously gives of CO_2 gas,
yielding a NAMETAG-peptide conjugate as the final
product. The NAMETAG portion can contain heavy
isotopic substitution, as exemplified in Figure 2.

Figure 4A shows the structure of a VICAT
reagent having a radioactive acetyl group as the
detectable moiety (^{14}C-Acetyl-VICAT). Figure 4B shows
a reaction scheme for the synthesis of ^{14}C-Acetyl-
VICAT.

Figure 5 shows a synthetic scheme for a VICAT
reagent with a ^{14}C-methyl group.

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Figure 6 shows a synthetic scheme for an NBD-VICAT reagent.

Figure 7 shows quantification of recombinant human group V secreted phospholipase A_2 (hGV). Figure 7A (left panel) shows the abundance of the doubly charged y12 ion (the most intense y-ion, m/z = 800.8) for the light peptide derived from a $VICAT_{SH}$-tagged hGV protein as a function of capillary-LC retention time. Figure 7B (right panel) shows the doubly chared y12 ion for the heavy peptide (m/z = 803.8).

DETAILED DESCRIPTION OF THE INVENTION

The invention provides reagents and methods for the detection of specific proteins in a complex sample. The methods can be used to quantify the amount of protein in the sample. The method is based on the concurrent introduction of a detectable moiety, for example, a fluorescent reagent or a radiolabel, a stable isotope tag, and an affinity tag at specific sites in a protein. The detectable moiety is used for the visualization of tagged peptides after proteolysis of the tagged protein sample and peptide separation. The stable isotope tag is used for accurate quantification of the abundance of the protein in the sample using a mass spectrometer. The affinity tag is required for the isolation of the tagged peptides. The reagent having these properties is termed VICAT, for Visible Isotope-coded Affinity Tag. To avoid complications in analyzing tandem mass spectra of tagged peptides caused by the relatively large VICAT group, the reagents can contain a cleavable linker for the removal of a major portion of the VICAT group prior to mass spectrometric analysis. The reagents and methods of the invention allow the rapid, sensitive and

quantitative detection, as well as accurate absolute
quantification, of selected proteins contained in
complex samples.

A VICAT reagent of the invention has the
following general structure: i) a functional group that
specifically reacts with a chemical group in the target
molecule, which is typically a protein; ii) a stable
isotope tag that remains covalently attached to the
specific target molecule and peptides derived from the
target molecule; iii) a cleavable linker peripheral to
the isotope tag; iv) a detectable group; and v) an
affinity group.

The methods of the invention advantageously
allow the detection of one or several selected proteins
in complex samples and determination of their absolute
quantity. In general, the method is based on the
generation of labeled peptide standards for each target
protein, where the peptide standards are chemically
identical to, but isotopically distinguished from, the
VICAT labeled peptides generated by enzymatic digestion
of a protein sample mixture containing the target
protein. The standard peptides are generated by
chemical synthesis of the target peptide followed by
labeling of the peptide with one isotopic form of the
VICAT reagent. The standard peptides conjugated with a
VICAT reagent are purified and can be quantitatively
calibrated for use as a known standard.

Defined amounts of the labeled peptide
standards are added to the protease digest of the
sample, and the peptide mixture is separated by
isoelectric focusing (IEF) in a polyacrylamide gel or
in solution, for example, in a capillary. Other types
of peptide separation can also be used, for example,
reverse phase high pressure liquid chromatography

(HPLC) or other well known methods of peptide
separation, as disclosed herein. The location of the
tagged peptides in the gel or capillary or other form
of peptide separation is determined by fluorescence or
visual imaging of a similarly prepared isoelectric
focusing strip or capillary that contains only the
VICAT-tagged peptide standards, or by autoradioagraphy
or counting of radioactive fractions of the gel or
capillary if the detectable moiety of the VICAT reagent
is a radiolabel. The region of the sample-containing
strip where the tagged peptides are located is
extracted, and the tagged peptides are immobilized to
affinity isolate the tagged peptides. The peptides are
affinity isolated by contacting the tagged peptides
with a solid support containing the cognate binding
molecule of the affinity group of the VICAT reagent,
for example, using avidin or streptavidin to bind to
biotin on the VICAT reagent. Immobilized peptides are
recovered either by adding a competitive ligand for the
affinity group of the VICAT reagent, by changing the
solution conditions such that the VICAT-peptide
conjugate is released from the solid support, for
example, by changing pH, salt, chaotropic agents, and
the like, or by cleavage of a cleavable linker, leaving
the isotope tag covalently attached to the peptide.
The recovered peptides can be analyzed by mass
spectrometry and, if required, by tandem mass
spectrometry. Accurate protein quantification is
achieved by calculating the ratio of signal intensities
of the standard peptide and the differentially labeled
peptide of identical sequence generated by enzymatic
digestion of the protein sample.

     Although described above with the affinity
isolation step performed after a previous purification
step, it is understood that the affinity isolation step
of VICAT-labeled polypeptides can be performed at any

convenient step of the methods of the invention. For
example, the affinity isolation step can be performed
prior to resolution on an IEF gel or chromatographic
separation. Furthermore, the affinity isolation step
can be performed prior to digestion with a protease, if
desired. One skilled in the art can readily determine
a suitable order for carrying out steps of the methods
of the invention so long as VICAT-labeled peptides are
isolated.

The method of the invention involves the
separation of the peptide mixtures generated by the
digestion of the labeled protein samples. Among the
multitude of mature peptide separation methods
available, a particularly useful method is isoelectric
focusing in polyacrylamide gels with immobilized pH
gradients. This method is particularly useful for the
following reasons: i) it is robust, relatively
inexpensive, highly reproducible and easily
multiplexed. IEF strips covering different pH ranges
are commercially available; ii) the method has immense
separation power and the pH gradient and therefore the
range of maximal separation can be tuned; iii) the pI
of peptides can be reasonably accurately calculated
from the amino acid sequence. The position of a
specific target peptide in the gel can therefore be
predicted; and iv) peptide recovery is simple and
efficient.

The methods of the invention can be
advantageously used for the detection and accurate
absolute quantification for one or more particular
proteins in a complex protein sample. The methods for
detecting and quantitating proteins or other molecules
in a sample can be used for a variety of purposes. For
example, the methods of the invention can be used for
the detection and quantification of proteins coded for

by genetic disease genes in cell lysates or other protein extracts prepared from biopsies or body fluids for the purpose of diagnosis or prognosis. The methods of the invention are also useful for the detection and quantification of specifically modified forms of a protein. In addition, the methods of the invention can be used for the detection and quantification of sequence variants of a protein, including polymorphisms, splice isoforms and sequence variants created by post-translational processing. Furthermore, the methods of the invention can be used for the detection of a single or multiple marker proteins in a biological sample, including those that are diagnostic or prognostic for a specific disease. The methods of the invention are advantageous in allowing quantification of the absolute amounts of multiple proteins in a complex sample without the need for specific reagents such as antibodies and can be carried out within a simplified operation.

Moreover, because the methods can be used quantitatively, the methods of the invention can be used for the detection of changes in abundance of specific proteins in complex mixtures. For example, the detection of proteins that are aberrantly expressed in disease states, genetic or otherwise, that is, the detection of marker proteins, is common in clinical testing and diagnosis. Commonly, such tests are based on some form of immunodetection, for example, western blotting, ELISA, of the target protein(s), which requires one or several antibodies specific for the target protein, and typically one protein is measured per assay. In contrast, the methods of the invention are advantageous in that they allow the detection of changes in the abundance of a selected single protein or constellations of multiple proteins in complex samples.

The methods of the invention incorporate a
modification of the previously described ICAT-based
proteomics technology (Gygi et al., <u>Nat. Biotechnol.</u>
17:994-999 (1999); WO 00/11208, each of which is
incorporated herein by reference). The use of an ICAT-
type reagent is advantageous for detecting one or
several proteins of known identity in a complex protein
mixture and can be greatly simplified by the ability to
readily prepare an internal standard that is chemically
identical, but isotopically-distinguished, to the
tagged peptides derived from the proteins of interest.
The methods of the invention using a VICAT-type reagent
advantageously allow the ready detection of the protein
of interest using a detectable group such as a
fluorophore or radiolabel. Thus, an ICAT-type reagent
can be modified to contain a fluorophore, a visible
dye, or radiolabel so that the internal standard can be
readily detected and quantitatively calibrated either
fluorimetrically or simply by eye in the case of
colored fluorophores or dyes, or by autoradiography,
liquid scintiallation counting or Cerenkov counting in
the case of radiolabels. Since the target proteins are
generally characterized using high-resolution
chromatographic separation of peptides prior to their
detection by ESI-MS, the reagents of the invention
allow the chromatographic position of the tagged
peptides of interests to be readily visualized.

Thus, Visible ICAT reagents (VICAT reagents)
are prepared having the properties of an ICAT reagent
(Gygi et al., *supra*, 1999; WO 00/11208). The VICAT-
type reagent also contains a detectable moiety such as
a fluorophore or radiolabel. A VICAT-type reagent also
can include a cleavable linker. The inclusion of a
cleavable linker allows release of a significant
portion of the VICAT reagent after chromatographic

separation and visualization of the VICAT-tagged
protein of interest. After cleavage, a small,
isotopically substituted fragment of the VICAT reagent
remains covalently attached to the protein or peptide
of interest. This allows the peptide from which the
bulk of the VICAT reagent has been removed to be
analyzed by tandem ESI-MS with the ion fragmentation
directed in the peptide rather than in the tag.
Peptide-directed fragmentation allows for peptide
sequencing and thus peptide identification.

The reagents and methods of the invention
have widespread application in quantitative
biochemistry and proteomics. Among the advantages of
the methods of the invention are the potential ability
to detect and quantify multiple peptides in the same
analysis (multiplexing), very precise quantification,
and the lack of need for peptide-specific antibodies or
other reagents.

The invention provides a VICAT reagent
comprising an affinity tag, a detectable moiety, such
as a fluorescent or non-fluorescent dye or radiolabel,
a linker, which can be cleavable, a stable isotope tag,
and a reactive group. The VICAT reagent is used to tag
a molecule such as a polypeptide to modify the molecule
with specific functionalities. In particular, the
reactive group is a chemical moiety having reactivity
for a chemical moiety on the target molecule. Thus,
the reactive group couples the VICAT reagent to a
target molecule. Once tagged with a VICAT reagent, the
target molecule is attached to an affinity tag, a
detectable moiety, a linker and an isotope tag. The
affinity tag can be used to specifically isolate tagged
molecules. The detectable group is used to visualize
or locate tagged molecules. The linker serves to
bridge the affinity tag and detectable group to the

isotope tag. The linker can include a cleavable moiety
so that the tagged molecule can be released from the
affinity tag and detectable moiety. For example, if
the affinity tag is used to affinity isolate tagged
molecules on a solid support, the linker can be cleaved
to release the affinity isolated tagged molecules.

The isotope tag is a chemical moiety that can
be used to incorporate heavy isotopes, allowing the
generation of multiple forms of a chemically identical
but isotopically distinct moiety. Differential isotope
tags are particularly useful for quantitative analysis
since a sample molecule can be compared directly to a
standard molecule, with the only difference being the
differential isotope tag, thus allowing direct
comparison between the sample and standard molecule.
Due to the relative position of the functionalities of
a VICAT reagent of the invention, the VICAT reagent can
be used to affinity isolate peptides and release the
peptides from the affinity support while concurrently
transferring an isotope tag to the released peptides.
Incorporation of the isotope tag allows comparison to
standard peptides labeled with a differentially labeled
isotope tag for convenient qualitative and/or
quantitative analysis.

An exemplary VICAT reagent of the invention
can have the general formula:

$$A \diagdown$$
$$\diagup L\text{-}T\text{-}R$$
$$D \diagup$$
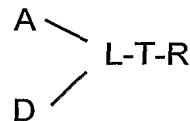
where A is an affinity tag, D is a detectable moiety,
such as a fluorescent dye or radiolabel, L is a linker,
which can be cleavable, T is an isotope tag, and R is a

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reactive group having a chemical functionality suitable
for covalently coupling to a target molecule such as a
protein. The formula above is representative of the
VICAT reagents shown in Figures 1, 2 and 4. It is
understood that any arrangement of the functionalities
of the VICAT reagent can be employed so long as each of
the moieties can function as disclosed herein and
needed for a particular application. Generally, the
affinity tag and detectable moiety are positioned so
that the linker separates them from the isotope tag and
reactive group, allowing cleavage of the affinity tag
and detectable moiety from a tagged molecule. Thus, in
addition to the formula shown above, a reagent of the
invention can have the formula A-D-L-T-R or D-A-L-T-R,
or any arrangement that allows the affinity tag,
detectable moiety, linker, isotope tag, and reactive
group to function as desired.

The structures and synthetic schemes of
exemplary VICAT reagents are shown in Figures 1, 2 and
4. The reactive functional group depicted in Figures
1, 2 and 4 is an iodoacetyl group, which specifically
reacts with SH groups on reduced proteins. The
particular isotope tag group depicted is an N-
aminoethylacetamido group, also known as a NAMETAG
group. The linker shown is a cleavable linker
containing the photocleavable 2-(2-nitrophenyl)-ethan-
2-ol group. The dye group shown in Figure 1 is 7-
nitrobenz-2-oxa-1,3-diazole (NBD). The dye group shown
in Figure 2 is 5-carboxytetramethylrhodamine. The
affinity group shown is a biotin. Figure 3 shows how
the NAMETAG-peptide is formed after photocleavage of a
VICAT-peptide conjugate.

The VICAT reagents described above and
depicted in Figures 1, 2, 4, 5 and 6 are merely
exemplary of the reagent of the invention having an

affinity tag, a detectable moiety, a linker, and
isotope tag, and a reactive group, which can have the
general formulas described above. One skilled in the
art will readily recognize that many structural
variations of the structures shown in Figures 1, 2 and
4 would be suitable for a reagent of the invention and
would provide the above-described characteristics.

        In particular, an affinity tag of the VICAT
reagent is used so that, when the VICAT reagent is
attached to a molecule, the affinity tag can be used to
isolate the molecule from a complex mixture. As used
herein, an "affinity tag" refers to a chemical moiety
having binding activity for a capture moiety. One
skilled in the art can readily determine a suitable
affinity tag for use in a VICAT reagent of the
invention. The general features of an affinity tag
include being stable, relatively small, having high
affinity and specific binding activity to a cognate
binding partner, being unlikely to undergo non-specific
interactions and adsorption to vessels, and being
soluble in aqueous solution. Accordingly, suitable
criteria for selection of an affinity tag include but
are not limited to a desired size, solubility, affinity
and/or specificity for a particular capture moiety,
lack of non-specific binding, the nature of the
molecule to be captured, such as whether any of the
sample molecules would be able to bind to the capture
moiety, and the like. Any of these or other desirable
criteria can be considered by one skilled in the art
for affinity tag to be incorporated into a VICAT
reagent of the invention. An exemplary affinity tag is
biotin, and the corresponding capture moiety is avidin
or streptavidin. Biotin is particularly useful as an
affinity tag because it forms a high affinity
interaction with avidin or streptavidin and can

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therefore readily be used to affinity isolate molecules
that have reacted with the VICAT reagent.

In addition to biotin, other affinity tags
can be used (see WO 00/11208). For example, modified
biotin-based reagents can be used, including d-
iminobiotin, which bind to avidin/streptavidin.
Another useful biotin-based reagent is N-biotinyl-
sarcosine, which binds tightly to avidin/streptavidin
but can be released more easily than biotin (see Gerber
et al., Bioconj. Chem. 12:603-615 (2001)). Maltose can
also be used as an affinity tag since it binds to
maltose binding protein. Similarly, other sugars and
sugar binding molecules such as lectins are suitable as
an affinity tag/capture moiety pair. Another suitable
affinity tag/capture moiety pair includes glutathione,
which binds to glutathione-S-transferase. In addition,
a poly-His tag, generally containing about 5 to about
10 histidines, can be used to affinity purify a
molecule using metal chelate chromatography.

Other suitable affinity tags include an
epitope for which a specific antibody is available. An
epitope can be, for example, a short peptide of about
3-5 amino acids or more, a carbohydrate, a small
organic molecule, and the like. Epitope tags have been
used to affinity purify recombinant proteins and are
commercially available. For example, antibodies to
epitope tags, including myc, FLAG, hemaglutinin (HA),
green fluorescent protein (GFP), polyHis, and the like,
are commercially available (see, for example, Sigma,
St. Louis MO; PerkinElmer Life Sciences, Boston MA).
In addition, a hapten, such as a dinitrophenyl group,
can be used with an antibody specific for the hapten.
Methods for making antibodies against an epitope tag
are well known to those skilled in the art (Harlow and
Lane, Antibodies: A Laboratory Manual, Cold Spring

Harbor Laboratory Press (1988)). One skilled in the
art can readily determine suitable affinity tags for
isolation of molecules to which the VICAT reagent has
been attached.

Yet another suitable affinity tag is a 1,2-
diol, such as 1,2-dihyroxyalkanes, including those of
cyclic alkanes, for example, 1,2-dihydroxycyclohexane,
which bind to an alkyl or aryl boronic acid or boronic
acid esters , such as phenyl-B(OH)$_2$ or hexyl-
B(OEthyl)$_2$, which can be attached via the alkyl or aryl
group to a solid support material, such as agarose. A
nitrilotriacetic acid group can also be used as an
affinity tag (WO 00/11208).

The above-described affinity tags can
interact non-covalently with the corresponding capture
moiety. Alternatively, an affinity tag can interact
covalently with a capture moiety. In such a case,
either the affinity tag or the capture moiety, in
addition to containing chemical characteristics
providing a specific interaction, can contain a
reactive group capable of reacting covalently to join
the affinity tag and capture moiety. In addition, a
cross-linking agent can be used to covalently bind a
non-covalent affinity interaction. Such a covalent
interaction would allow stringent wash conditions, and
the presence of a cleavable linker in the VICAT reagent
would allow release and tagging of the VICAT-labeled
molecule(s), as disclosed herein.

Furthermore, it is understood that the
affinity tag of the VICAT reagent need not be distinct
from other moieties. If a capture moiety is available
for another moiety of a VICAT reagent, then one moiety
of the VICAT reagent can serve two functions. For
example, antibodies are available against the

detectable moiety fluorescein, and fluorescein can thus function as both an affinity tag and detectable moiety. Similarly, other moieties can have multiple functions in a VICAT reagent so long as each of the functions can work independently.

The VICAT-type reagents of the invention also include a detectable moiety. As used herein, a "detectable moiety" refers to a moiety that imparts a physicochemical property such that the moiety can be detected. Such a detectable moiety allows a VICAT-tagged molecule to be visualized. The detectable moiety is generally a moiety that allows convenient detection of the tagged molecule, for example, a fluorescent, a colored, or a colorimetric moiety. Similar to the desired characteristics of an affinity tag, the detectable moiety is generally stable, relatively small, is unlikely to undergo non-specific interactions and adsorption to vessels, and is soluble in aqueous solution. In the case of a fluorescent moiety, it is particularly useful for the moiety to have high quantum yield, a high absorption coefficient, and a quantum yield that is not solvent dependent. Accordingly, suitable criteria for selection of a detectable moiety include but are not limited to a desired size, solubility, lack of non-specific binding, desired method of detection including speed and convenience of detection, and the like. In the case of fluorescent detectable moieties, the criteria can include quantum yield and properties related to solvent, absorption coefficient, excitation and emission wavelengths, fluorescence lifetime, and the like. Any of these or other desirable criteria can be considered by one skilled in the art for affinity tag to be incorporated into a VICAT reagent of the invention.

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Thus, the detectable group can be a
fluorophore, such as rhodamine-type fluorophore
depicted in Figure 2. Other suitable fluorescent
moieties are well known to those skilled in the art
(see Hermanson, Bioconjugate Techniques, pp. 297-364,
Academic Press, San Diego (1996); Molecular Probes,
Eugene OR). Rhodamine derivatives include, for
example, tetramethylrhodamine, rhodamine B, rhodamine
6G, sulforhodamine B, Texas Red (sulforhodamine 101),
rhodamine 110, and derivatives thereof such as
tetramethylrhodamine-5- (or 6), lissamine rhodamine B,
and the like. Other suitable fluors for a VICAT
reagent include 7- nitrobenz-2-oxa-1,3-diazole
(NBD)(see Figure 1).

Additional exemplary fluorophores include,
for example, fluorescein and derivatives thereof.
Other flurophores include napthalenes such as dansyl
(5-dimethylaminonapthalene-1-sulfonyl). Additional
fluorophores include coumarin derivatives such as 7-
amino-4-methylcoumarin-3-acetic acid (AMCA), 7-
diethylamino-3-[(4'-(iodoacetyl)amino)phenyl]-4-
methylcoumarin (DCIA), Alexa fluor dyes (Molecular
Probes), and the like.

Other fluorophores include 4,4-difluoro-4-
bora-3a,4a-diaza-s-indacene (BODIPY™) and derivatives
thereof (Molecular Probes; Eugene OR). Further
fluorophores include pyrenes and sulfonated pyrenes
such as Cascade Blue™ and derivatives thereof,
including 8-methoxypyrene-1,3,6-trisulfonic acid, and
the like. Additional fluorophores include
pyridyloxazole derivatives and dapoxyl derivatives
(Molecular Probes). Additional fluorophores include
Lucifer Yellow (3,6-disulfonate-4-amino-naphthalimide)
and derivatives thereof. CyDye™ fluorescent dyes

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(Amersham Pharmacia Biotech; Piscataway NJ) can also be used.

In general, the detectable moiety is a relatively small organic molecule such as the fluorophores described above. However, it is understood that fluorescent proteins can also be used so long as the size of the fluorescent protein does not interfere with the other functions of the VICAT reagent or the needs of the particular experiment. Thus, other fluorescent moieties include phycobiliproteins and derivatives such as phycoerythrin and phycocyanin from various species, as well as green fluorescent protein and derivatives thereof or other fluorescent proteins.

The detectable moiety can also be a chromophore. Exemplary chromophores include, for example, phenolphthalein, malachite green, nitroaromatics such as nitrophenyl, diazo dyes, dabsyl (4-dimethylaminoazobenzene-4'-sulfonyl), and the like (see Hermanson, *supra*, 1996).

Although the detectable moiety is described above as a group that allows ready visualization of the VICAT-tagged molecule, it is understood that any moiety that imparts a detectable property can be used as a detectable moiety. Thus, a detectable moiety can be a radioactive moiety, which can be visualized by autoradiography, fluorography or phosphorimaging, or can be detected by scintillation or Cerenkov counting of chromatographic fractions, polyacrylamide gel or gel slices, or extracts of regions of a polyacrylamide gel. Suitable radioactive isotopes for use in a detectable moiety include, for example, ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{125}I, and the like. Thus, a radioactive detectable moiety can include a chemical group that contains hydrogen, carbon, phosphorus, sulfur, iodine, and the like

incorporated as a radioactive isotope. Exemplary
radioactive detectable moieties are shown in Figures 4B
and 5. In addition, the detectable moiety can be
chemiluminescent, can contain spin labels, can be
detectable with ultraviolet light, or can be other
detectable groups.

It is understood that, when a detectable
moiety is a small moiety such as a radioactive atom,
the detectable moiety can be incorporated into one of
the other functional groups of a VICAT reagent. For
example, a radioactive moiety can be attached to a
linker as a separate chemical moiety or can be
incorporated into the linker by using a radioactive
atom in the linker. Similarly, one or more radioactive
atoms can be incorporated into the affinity tag,
isotope tag or reactive group. In such a case, the
detectable moiety can be positioned so that it is
removed upon cleavage of the linker. However, since
the detectable moiety is relatively small, it can be
positioned so that the detectable moiety is retained on
the labeled polypeptide after cleavage of the linker
and release from an affinity capture reagent. An
exemplary radioactive VICAT reagent having a ^{14}C-acetyl
group is depicted in .Figure 4. Another exemplary
radioactive VICAT reagent having a ^{14}C-methyl group is
depicted in Figure 5.

The "linker" of a VICAT reagent is used to
bridge the affinity tag and detectable moiety to the
isotope tag and reactive group. The linker is
generally a cleavable group. A cleavable group is
particularly useful when mass spectrometry (MS) is
being used as an analytical method. Because the
affinity tag and detectable tag can be large moieties,
it is useful to have the option of cleaving off these
large groups from the molecules to be analyzed by MS.

However, it is understood that cleavage of a cleavable
linker or the use of a cleavable linker is not required
so long as the groups of the VICAT reagent retained on
the tagged molecule are compatible with the analytical
method being used. Any of a number of chemical groups
can be used to bridge the affinity tag and detectable
moiety with the isotope tag and reactive group.
Suitable criteria for selection of a linker include but
are not limited to a desired size, solubility, ability
to cleave or not cleave the linker, the type of
cleavage method to be used, and the like. Any of these
or other desirable criteria can be considered by one
skilled in the art for affinity tag to be incorporated
into a VICAT reagent of the invention.

A cleavable linker can be a photocleavable or
chemically cleavable moiety. The photocleavable linker
2-(2-nitrophenyl)-ethan-2-ol is depicted in Figures 1,
2, 4, 5 and 6. Other suitable photocleavable linkers
include, for example, linkers containing o-nitrobenzyl,
desyl, trans-o-cinnamoyl, m-nitrophenyl, benzylsulfonyl
groups (see, for example, Dorman and Prestwich, Trends
Biotech. 18:64-77 (2000); Greene and Wuts, Protective
Groups in Organic Synthesis, 2nd ed., John Wiley &
Sons, New York (1991); U.S. Patent Nos. 5,143,854;
5,986,076; 5,917,016; 5,489,678; 5,405,783). One
skilled in the art will readily recognize that these
exemplary photocleavable groups as well as others can
be used in a VICAT reagent.

In addition to a photocleavable linker, a
chemically cleavable linker can also be used.
Exemplary cleavable linkers can contain a disulfide,
which can be cleaved with reducing agents; a diol,
which can be cleaved with periodate; a diazo bond,
which can be cleaved with dithionate; an ester, which
can be cleaved with hydroxylamine; and a sulfone, which

can be cleaved with base (see Hermanson, <u>Bioconjugate</u>
<u>Techniques</u>, Academic Press, San Diego (1996); Pierce
Chemical Co., Rockford IL). Acid cleavable groups can
also be used.

Furthermore, a linker cleavable by an enzyme
can be used. For example, a protease can be used to
cleave a cleavable linker having a suitable recognition
sequence for the protease. Particularly useful
proteases are endopeptidases such as factor Xa, tobacco
etch virus (TEV) protease, trypsin, chymotrypsin,
Staphylococcus aureus protease, submaxillaris protease,
and the like. The protease can be selected based on
the incorporation of a particular cleavable recognition
sequence into the linker. Other considerations for
selecting a protease include the presence or absence of
a recognition sequence in the molecule being captured
and released.

For example, a rare cleaving protease such as
TEV protease or factor Xa can be used to cleave a
functional group containing the corresponding protease
recognition sequence, resulting in release of the
captured molecule. Such rare cleaving proteases are
particularly useful for releasing an intact polypeptide
molecule since the recognition sequence for these
proteases would not occur in the vast majority of
polypeptides. Alternatively, a polypeptide sample can
be treated with a specific protease, and the digested
peptides isolated by the methods disclosed herein. In
such a case, the captured peptides would not contain a
recognition sequence for the protease used for cleavage
since the polypeptide has already been digested. In
addition, if desired, an intact polypeptide can be
captured and digested with a protease after binding to
the solid support, resulting in the incorporation and
release of a label on the peptide fragment of the

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polypeptide that was captured on the solid support.
Thus, protease digestion can be used before or after
capture of a sample molecule, as desired.

In addition to proteases, a cleavable
functional group can be a recognition sequence for an
endonuclease such as a restriction enzyme. Thus, an
appropriate recognition sequence for a restriction
enzyme can be incorporated as a cleavable functional
group and cleaved with the respective restriction
enzyme. It is understood that such a nucleotide
functional group can be useful for capturing and
releasing a nucleic acid or a polypeptide, or any other
type of molecule, as desired. Similarly, a protease
recognition sequence can be useful for capturing and
releasing a polypeptide, nucleic acid or any other type
of molecule, as desired.

A VICAT reagent of the invention also
contains a stable isotope tag. As used herein an
"stable isotope tag" refers to a chemical moiety having
suitable chemical properties for incorporation of a
stable isotope, allowing the generation of
differentially labeled reagents which can be used to
differentially tag a polypeptide in two samples. The
isotope tag, in combination with the linker, has a
sufficient length to allow the reactive group to bind
to a sample polypeptide and the affinity tag to bind to
its cognate binding partner. The isotope tag also has
an appropriate composition to allow incorporation of a
stable isotope at one or more atoms. A particularly
useful stable isotope pair is hydrogen and deuterium,
which can be readily distinguished using mass
spectrometry as light and heavy forms, respectively.
Any of a number of isotopic atoms can be incorporated
into the isotope tag so long as the heavy and light
forms can be distinguished using mass spectrometry, for

example, ^2H, ^{13}C, ^{15}N, ^{17}O, ^{18}O or ^{34}S. Differential
isotopic tags will generally differ by minimally 2 mass
units and up to about 20 mass units, for example, about
4 to about 16 mass units, about 6 to about 12 mass
units, and the like. Accordingly, the mass difference
can be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,
16, 17, 18, 19 or 20 mass units, or even a greater
number of mass units. One skilled in the art can
readily determine an appropriate mass differential
suitable for isotope tags for detecting mass
differences of VICAT-labeled molecules using mass
spectrometry. A multiple of a particular isotope can
be incorporated and/or a combination of isotopes (see
Figures 1 and 2).

     Suitable criteria for selection of an
affinity tag include but are not limited to a desired
size, solubility, the presence of a suitable type and
number of atoms for incorporation of desired heavy
isotope(s) sufficient for MS analysis, and the like.
Any of these or other desirable criteria can be
considered by one skilled in the art for affinity tag
to be incorporated into a VICAT reagent of the
invention.

     Exemplary isotope tags include the 4,7,10-
trioxa-1,13-tridecanediamine based linker and its
related deuterated form, 2,2',3,3',11,11',12,12'-
octadeutero-4,7,10-trioxa-1,13-tridecanediamine,
described by Gygi et al. (*supra*, 1999). Other
exemplary isotope tags include a diethylenediamine, as
shown in Figures 1, 2 and 4. One skilled in the art
can readily determine any of a number of appropriate
isotope tags useful in an VICAT-type affinity reagent
that satisfy the above-described criteria. As used
herein, the portion of the VICAT reagent which will
remain attached to a sample molecule upon cleavage of a

cleavable linker and which has sufficient chemical
characteristics to allow the incorporation of isotopic
atoms is considered to be an isotope tag. It is
understood that such a portion is considered an isotope
tag, regardless of whether different isotopes have been
incorporated into the tag. Thus, as shown in Figures 1
and 2, three VICAT reagents are depicted that are
differentially labeled in the isotope tag, one with no
heavy isotope and the others depicted as +6 and +12
forms.

Thus, an isotope tag can be an alkyl,
alkenyl, alkynyl, alkoxy, aryl, and the like; at least
one of the carbons can be replaced with O, S, N; can
contain O, S, NH, NR, NRR', CO, COO, COS, S-S, SO,
SO_2, SH, CO-NR', CS-NR', Si-O, aryl or diaryl group,
where R is an alkyl, alkenyl, alkynyl, alkoxy, or aryl
group and R' is hydrogen, an alkyl, alkenyl, alkynyl,
alkoxy or aryl group; and can be optionally
substituted, for example, with O, S, N, and the like,
and can contain an amine, carboxyl, sulfhydryl, and the
like (see WO 00/11208). An isotope tag is generally of
about 2 to about 100 atoms, although can be larger, for
example, about 2 to about 50 atoms, about 2 to about 40
atoms, about 2 to about 30 atoms, about 2 to about 20
atoms, or about to 2 to about 10 atoms in length. The
20 naturally occurring amino acids or non-naturally
occurring amino acids can also be used as isotope tags.

The above-described isotope tags and other
derivatives can be made in the same manner as that
disclosed herein using methods well known to those
skilled in the art. One skilled in the art will
readily recognize that a number of suitable chemical
groups can be used as an isotope tag so long as the
isotope tag can be differentially isotopically labeled.
One skilled in the art can readily determine a number

of suitable isotope tags of various sizes based on
criteria such as desired size of the tag, chemical
composition for incorporation of desired atom types and
number of differential isotopes, convenience of
chemical synthesis, chemical stability, and the like.
The isotope tag has at least a minimal size that allows
sufficient differential labeling of the isotope tag
suitable for analysis in a mass spectrometer. For
example, the isotope tag is selected so that a
sufficient mass differential can be detected in a mass
spectrometer, taking into account naturally occurring
isotope distribution (mostly ^{13}C) that can overlap with
the isotope tagged molecules if the mass differential
is small. One skilled in the art can readily recognize
these and other desirable characteristics of an isotope
tag.

The use of an isotope tag is particularly
useful for quantifying the amount of sample molecules
using mass spectrometry. The method is based on
derivatizing a specimen molecule such as a polypeptide
with a VICAT reagent. A control reference sample, for
example, synthesized known proteins or peptides or a
control sample, and a test sample are differentially
labeled with lighter and heavier forms of the VICAT
affinity reagent. For quantitative analysis, the
derivatized samples can be combined and analyzed in
parallel using mass spectrometry, as described below
(see Gygi et al., *supra*, 1999).

A VICAT reagent of the invention also has a
"reactive group," which is reactive with a chemical
moiety on a molecule in a sample and therefore can be
covalently coupled to a molecule in a sample such as a
polypeptide. Reactive groups are well known to those
skilled in the art (see, for example, Hermanson, *supra*,
1996; Glazer et al., Laboratory Techniques in

Biochemistry and Molecular Biology: Chemical
Modification of Proteins, Chapter 3, pp. 68-120,
Elsevier Biomedical Press, New York (1975); Pierce
Catalog, Pierce, Rockford IL). Any of a variety of
reactive groups can be incorporated into a VICAT type
affinity reagent so long as the reactive group can be
covalently coupled to a polypeptide or other desired
molecule in a sample. The reactive group can also be a
group that reacts with a moiety on a target sample
molecule in the presence of an extrinsic reagent such
as a crosslinking reagent. For example, bis-maleimide
can be used to crosslink an SH group on a peptide with
an SH group on the VICAT reagent. These and other
cross-linking reagents suitable for coupling a peptide
to a VICAT reagent are well know to those skilled in
the art (see, for example, Hermanson, *supra*, 1996;
Glazer et al., *supra*, 1975; Pierce).

Suitable criteria for selection of a reactive
group include but are not limited to the type of
molecule to be targeted with the VICAT reagent, the
particular chemical moiety on the target molecule to be
reacted, the stability of the reactive group under the
buffer conditions used for the reaction, and the like.
Any of these or other desirable criteria can be
considered by one skilled in the art for affinity tag
to be incorporated into a VICAT reagent of the
invention.

For example, a polypeptide can be coupled to
the ICAT type affinity reagent via a sulfhydryl
reactive group, which can react with free sulfhydryls
of cysteine or reduced cystines in a polypeptide. An
exemplary sulfhydryl reactive group includes an
iodoacetamido group (see Gygi et al., *supra*, 1999).
Other exemplary sulfhydryl reactive groups include
maleimides, alkyl and aryl halides, haloacetyls, α-

haloacyls, pyridyl disulfides, aziridines, acrylolyls, arylating agents and thiomethylsulfones. If desired, the polypeptides can be reduced, for example, with tri-butylphosphine, dithiothreitol, mercaptoethanol, and the like, prior to reacting with a VICAT type reagent, which is particularly useful when the VICAT reagent contains a sulfhydryl reactive group. In addition, amino groups can be converted to sulfhydryl groups by chemical modification so that a sulfhydryl reactive VICAT reagent, such as VICAT$_{SH}$, can be reacted with the modified peptides (see Example VII).

A reactive group can also react with amines such as the α-amino group of a peptide or the ϵ-amino group of the side chain of Lys, for example, imidoesters, N-hydroxysuccinimidyl esters (NHS), isothiocyanates, isocyanates, acyl azides, sulfonyl chlorides, aldehydes, ketones, glyoxals, epoxides (oxiranes), carbonates, arylating agents, carbodiimides, anhydrides, and the like. In addition, a vinyl sulfone such as $R-SO_2-CH=CH_2$ or $R-NHSO_2-CH=CH_2$, which can undergo a nucleophilic Michael-type addition reaction, can be used to react with sulfhydryl or amine groups on the target molecule.

A reactive group can also react with carboxyl groups found in Asp or Glu or the C-terminus of a peptide, for example, diazoalkanes, diazoacetyls, carbonyldiimidazole, carbodiimides, and the like. A reactive group that reacts with a hydroxyl group includes, for example, epoxides (oxiranes), carbonyldiimidazoles, N,N'-disuccinimidyl carbonates, N-hydroxycuccinimidyl chloroformates, and the like. A reactive group can also react with amino acids such as histidine, for example, α-haloacids and amides; tyrosine, for example, nitration and iodination; arginine, for example, butanedione, phenylglyoxal, and

nitromalondialdehyde; methionine, for example,
iodoacetic acid and iodoacetamide; and tryptophan, for
example, 2-(2-nitrophenylsulfenyl)-3-methyl-3-
bromoindolenine (BNPS-skatole), N-bromosuccinimide,
formylation, and sulfenylation (Glazer et al., *supra*,
1975). In addition, a reactive group can also react
with a phosphate group for selective labeling of
phosphopeptides (Zhou et al., Nat. Biotechnol., 19:375-
378 (2001)), or with other covalently modified
peptides, including glycopeptides, lipopeptides, or any
of the known covalent polypeptide modifications. One
skilled in the art can readily determine conditions for
modifying sample molecules by using various reagents,
incubation conditions and time of incubation to obtain
conditions optimal for modification of sample molecule
for use in reagents and methods of the invention.

     The reactive groups described above can form
a covalent bond with the target sample molecule.
However, it is understood that a VICAT reagent can
contain a group that can non-covalently interact with a
sample molecule so long as the interaction has high
specificity and affinity.

     A VICAT reagent of the invention can contain
any combinations of the above-described affinity tags,
detectable moieties, linkers, isotope tags, and
reactive groups, as well as others well known to those
skilled in the art. For example, a VICAT reagent can
contain the affinity tag biotin, the detectable moiety
rhodamine, the photolabile linker 2-
nitrobenzyloxycarbonyl, the isotope tag
ethylenediamine, and the reactive group iodacetyl, as
depicted in Figure 2. Another VICAT reagent can
contain the affinity tag biotin, the detectable moiety
7-nitrobenz-2-oxa-1,3-diazole (NBD), the photolabile
linker 2-nitrobenzyloxycarbonyl, the isotope tag

ethylenediamine, and the reactive group iodacetyl, as
depicted in Figure 1.   The reagent depicted in Figure 1
is particularly useful because it is easy to make, is
stable, and has solubility properties in aqueous
solution sufficient for quantitative proteomics
analysis.   An additional VICAT reagent can contain the
affinity tag biotin, the radioactive detectable moiety
^{14}C-acetyl, the isotope tag ethylenediamine, and the
reactive group iodacetyl, as depicted in Figure 4.   An
additional VICAT reagent can contain the affinity tag
biotin, the radioactive detectable moiety ^{14}C-methyl,
the isotope tag ethylenediamine, and the reactive group
iodacetyl, as depicted in Figure 5.

Still another VICAT reagent can contain the
affinity tag glutathione, the detectable moiety
coumarin, a linker containing a benzylsulfonyl group,
the isotope tag tridecanediamine, and an isothiocyanate
reactive group.   Yet another VICAT reagent can contain
the affinity tag poly-His with six histidines, the
detectable moiety BODIPY, a linker containing a
disulfide, the isotope tag dodecane, and a
disuccinimidyl carbonate reactive group.

The above-described VICAT reagents are merely
exemplary of the VICAT reagents that can be readily
prepared by one skilled in the art in the same manner
as that disclosed herein using well known methods of
chemical synthesis, including methods similar to those
exemplified herein (see Example I).   Based on the
desired application of the VICAT reagent, one skilled
in the art can select a suitable affinity tag,
detectable moiety, linker, isotope tag, and reactive
group sufficient for desired criteria, as disclosed
herein.

The VICAT reagents of the invention can be used to label molecules in a sample. The methods are particularly useful for labeling polypeptides in a sample, and can thus be used for qualitative and quantitative proteome analysis. Although labeling of polypeptides is exemplified herein, it is understood that sample molecule having chemical properties reactive with the VICAT reagent reactive group can be labeled with a VICAT reagent of the invention. For example, a VICAT reagent can be used to label nucleic acids, carbohydrates, lipids, metabolites or other sample molecules.

The invention also provides a method of isolating a polypeptide in a sample. The method can include the steps of contacting a sample with a VICAT reagent of the invention under conditions allowing the reactive group to bind to and react with one or more polypeptides in the sample, thereby tagging one or more polypeptides with the reagent; resolving the polypeptides in the sample; visualizing the polypeptides tagged with the reagent; contacting the tagged polypeptides with a capture moiety for the affinity tag; and isolating the tagged polypeptides. It is understood that the steps of this method and other methods of the invention can be performed in any order so long as a VICAT reagent tags a sample molecule and can be used to isolate a target molecule. For example, an affinity isolation step of a VICAT-labeled molecule can be performed before or after other fractionation steps used to resolve molecules in a sample and can be performed before or after a visualization step. Furthermore, a sample can be resolved using various fractionation methods prior to labeling with a VICAT reagent. One skilled in the art can readily determine an appropriate order for carrying

out steps of the methods of the invention suitable for tagging and isolating a sample molecule.

As used herein, the term "polypeptide" refers to a peptide or polypeptide of two or more amino acids. A polypeptide can also be modified by naturally occurring modifications such as post-translational modifications, including phosphorylation, fatty acylation, prenylation, sulfation, hydroxylation, acetylation, addition of carbohydrate, addition of prosthetic groups or cofactors, formation of disulfide bonds, proteolysis, assembly into macromolecular complexes, and the like.

The invention provides a method of labeling a polypeptide in a sample by contacting a sample with a VICAT reagent of the invention under conditions allowing the reactive group to bind to one or more polypeptides in the sample. One skilled in the art can readily determine appropriate conditions so that the reactive group of a VICAT reagent can react with a sample molecule, including appropriate buffers, salts, pH, temperature, and the like (see Hermanson, *supra*, 1996). Thus, a VICAT reagent of the invention can be used to label a sample molecule via the reactive group, resulting in the attachment of the VICAT reagent and corresponding isotope tag to a sample molecule.

Once a VICAT reagent has reacted with a sample molecule, the sample is labeled in a manner that allows convenient visualization of the labeled molecule(s). For example, any of the visualization methods disclosed herein can be used to conveniently locate the labeled molecules. Such a visualization is particularly useful for locating a labeled sample molecule when a complex sample molecule has been resolved from other sample molecules. As used herein,

"resolving" when used in reference to a sample molecule refers to using a purification or fractionation step that separates the sample molecule from at least one other molecule in the sample. Thus, the method can further include the step of visualizing the molecules tagged with the VICAT reagent.

As used herein, the term "sample" is intended to mean any biological fluid, cell, tissue, organ or portion thereof, that includes one or more different molecules such as nucleic acids, polypeptides, or small molecules. A sample can be a tissue section obtained by biopsy, or cells that are placed in or adapted to tissue culture. A sample can also be a biological fluid specimen such as blood, urine or saliva. A sample can additionally be a cell extract from any species, including prokaryotic and eukaryotic cells as well as viruses. A tissue sample can be further fractionated, if desired, to a fraction containing particular cell types.

Furthermore, the sample can be fractionated by a number of known fractionation techniques. Since such fractionation methods separate molecules, such techniques are used to resolve sample molecules. Methods for resolving sample molecules are well known to those skilled in the art. Fractionation methods, which can be used to resolve sample molecules, include but are not limited to subcellular fractionation or chromatographic techniques such as ion exchange, including strong and weak anion and cation exchange chromatography, hydrophobic and reverse phase, size exclusion, affinity, hydrophobic charge-induction chromatography, dye-binding, and the like (Ausubel et al., Current Protocols in Molecular Biology (Supplement 56), John Wiley & Sons, New York (2001); Scopes, Protein Purification: Principles and Practice, third

edition, Springer-Verlag, New York (1993); Burton and
Harding, J. Chromatogr. A 814:71-81 (1998)). Other
fractionation methods include, for example,
centrifugation, electrophoresis, the use of salts, and
the like (see Scopes, *supra*, 1993).

Affinity chromatography can also be used
including, for example, dye-binding resins such as
Cibacron blue, substrate analogs, including analogs of
cofactors such as ATP, NAD, and the like, ligands,
specific antibodies useful for immuno-affinity
isolation, either polyclonal or monoclonal, and the
like. An exemplary affinity resin includes affinity
resins that bind to specific moieties that can be
incorporated into a polypeptide such as an avidin resin
that binds to a biotin tag on a sample molecule labeled
with a VICAT reagent of the invention, as disclosed
herein. The resolution and capacity of particular
chromatographic media are known in the art and can be
determined by those skilled in the art. The usefulness
of a particular chromatographic separation for a
particular application can similarly be assessed by
those skilled in the art.

Those of skill in the art will be able to
determine the appropriate chromatography conditions for
a particular sample size or composition and will know
how to obtain reproducible results for chromatographic
separations under defined buffer, column dimension, and
flow rate conditions. The fractionation methods can
optionally include the use of an internal standard for
assessing the reproducibility of a particular
chromatographic application or other fractionation
method. Appropriate internal standards will vary
depending on the chromatographic medium or the
fractionation method used. Those skilled in the art
will be able to determine an internal standard

applicable to a method of fractionation such as
chromatography.

Electrophoresis, including gel
electrophoresis or capillary electrophoresis, can also
be used to resolve sample molecules. As disclosed
herein, isoelectric focusing (IEF) is a particularly
useful method to resolve sample polypeptides. As
discussed herein, sample molecules can be processed,
for example, by protease cleavage into peptide
fragments. Accordingly, when referring to sample
molecules, the sample molecules can be intact as found
in an original sample or can be processed, for example,
into smaller molecules such as peptides from a
polypeptide sample.

If desired, sample molecules can be modified,
either before or after a fractionation step. For
example, the methods of the invention are particularly
useful for mass spectrometry (MS) analysis. In the
case of MS analysis of polypeptides, it is often useful
to cleave the polypeptide into smaller fragments, for
example, by proteolysis. Thus, a polypeptide molecule
can be enzymatically cleaved with one or more proteases
into peptide fragments. Exemplary proteases useful for
cleaving polypeptides include trypsin, chymotrypsin,
pepsin, papain, *Staphylococcus aureus* (V8) protease,
and the like. Polypeptides can also be cleaved
chemically, for example, using CNBr, acid or other
chemical reagents.

For polypeptide fragmentation, the
polypeptides in the sample mixture, or the polypeptides
contained in each fraction if optional sample
fractionation is employed, can be subjected to sequence
specific cleavage, such as cleavage by trypsin. The
use of sequence specific cleavage can be particularly

useful because the termini of peptides cleaved by a
sequence specific method can act as a constraint.
However, it is understood that the cleavage method used
to generate fragments need not be sequence specific, if
desired.

Furthermore, for polypeptide tagging, the
polypeptides in the sample can be denatured and
optionally reduced. Reducing the sample can be
particularly useful when the reactive group on the
VICAT reagent is reactive with a thiol. Other useful
reactive groups include amino or carboxyl groups of
polypeptides or specific post-translational
modifications, including phosphate, carbohydrate or
lipid.

The methods of the invention involve the
coupling of a VICAT-type reagent to a sample molecule,
which results in the incorporation of a visible tag,
which is detectable as disclosed herein. Such a
visible tag is particularly useful for visualizing the
location of a sample molecule resolved by a
fractionation method. Thus, in a complex sample, the
visible tag allows the convenient localization of a
VICAT-labeled molecule. Furthermore, as disclosed
herein, a specific sample molecule can be identified by
preparing known standards and running the standards in
parallel or in the same sample to facilitate the
visualization of a particular desired sample molecule.
If the standard molecules are added to the sample
molecules, a relatively high amount of the standard can
be added to indicate the position of the corresponding
target molecule of interest.

The ability to readily visualize a VICAT-
labeled molecule facilitates further characterization
of the labeled molecule, regardless of the complexity

of the original sample. In a complex sample, it is
likely that a number of sample molecules will be
labeled by the reactive group of the VICAT reagent.
For example, the methods of the invention are
particularly useful for proteomics analysis, where the
sample molecules to be analyzed are polypeptides or
peptide fragments thereof. By running a sample in
parallel with a known standard, a particular sample
molecule can be readily visualized. A known sample
molecule can be synthesized, labeled with a VICAT
reagent, and run in parallel under essentially the same
conditions as the sample molecules. For example, a
known polypeptide can be synthesized, optionally added
to an unlabeled sample, and resolved in the same manner
as the VICAT-labeled sample. Since the samples
molecules are run under similar conditions, the
location of the known polypeptide, also tagged with the
VICAT reagent, can readily be determined in the
parallel sample. Alternatively, a known molecule can
be synthesized and labeled with the same VICAT reagent,
but having a different detectable moiety, and run
together with the sample molecules. In such a case,
the different detectable moiety would have essentially
the same resolution as the other detectable moiety in
the particular fractionation method used. An example
of this is the use of a radiolabel as the detectable
moiety. The radiolabeled standard is added to the
sample, and the position of the analyte to be analyzed
is detected by autoradiography, fluorimetry, phospor
storage imaging, or liquid scintillation or Cerenkov
counting.

     Furthermore, a VICAT-labeled standard
molecule can be used to calibrate a particular
separation method so that the position of a
corresponding VICAT-labeled sample molecule can be
predicted when run under substantially similar

conditions. Because a separation method can be
performed reproducibly when run under substantially
similar conditions, a VICAT-labeled standard molecule
can be run on a separation medium under defined
conditions so that the location of the VICAT-labeled
standard molecule can be used to predict the location
of a VICAT-labeled sample molecule run under the same
defined conditions. This obviates the need to add the
standard molecule to the sample or to run the standard
molecule in parallel. Such an approach can be
particularly useful for analysis by IEF.

Once a VICAT-labeled molecule of interest has
been visualized and located in the separation medium,
the VICAT-labeled molecule can be isolated by
contacting with a capture moiety corresponding to the
affinity tag of the VICAT reagent. Alternatively, the
VICAT-labeled molecule can be affinity isolated by the
capture moiety prior to resolution on a separation
medium or visualization. For example, if the sample
molecules are resolved by IEF electrophoresis, the
region containing the VICAT-labeled sample molecule of
interest can be extracted and contacted with an
appropriate capture moiety (see Example III). Thus, by
visualizing the resolved sample molecules to locate a
VICAT-labeled molecule, the VICAT-labeled molecule of
interest can be further processed and affinity purified
in the absence of other VICAT-labeled molecules.

The capture moiety can be attached, for
example, to a bead, resin, membrane or disk, or can be
coupled to any type of solid support suitable for the
isolation of the VICAT-labeled molecule(s). It is
understood that the affinity tag can bind to a capture
moiety directly bound to the solid support or to a
capture moiety that binds via a secondary agent to the
solid support. For example, the capture moiety can be

bound in solution to the VICAT-labeled molecule and
subsequently bound to a solid support, such as by
binding to an antibody specific for the capture moiety.
One skilled in the art can readily determine a desired
method to affinity isolate a VICAT-labeled molecule
using well known affinity isolation methods.

The invention thus provides a method of
isolating one or more polypeptides in a sample. The
method can include the steps of contacting a sample
with a VICAT reagent of the invention under conditions
allowing the reactive group to bind to and react with
one or more polypeptides in sample, thereby tagging one
or more polypeptides with the reagent; resolving the
polypeptides in the sample; visualizing the
polypeptides tagged with the reagent; contacting the
tagged polypeptides with a capture moiety for the
affinity tag; and isolating the tagged polypeptides.
In addition to labeling polypeptides, this and other
methods of the invention can be used to label other
types of sample molecules such as carbohydrates,
nucleic acids, and the like, if desired. Since the
linker of the VICAT reagent can be cleavable, the
method can further include the step of cleaving the
linker. The cleavage results in the release of the
VICAT-labeled molecule such that the molecule retains
the isotope tag but is separated from the detectable
moiety and affinity tag. Thus, the VICAT-labeled
molecules can be readily released from an affinity
resin or other solid support to which the labeled
molecules are bound.

The isolated VICAT-labeled molecules can be
further characterized, if desired. A particularly
useful method for characterizing sample molecules is
mass spectrometry (MS), which can be used to identify
and/or quantify the VICAT-labeled molecule. Thus, the

invention provides a method of identifying a
polypeptide in a sample. The method can include the
steps of contacting a sample with a VICAT reagent of
the invention under conditions allowing the reactive
group to bind to and react with one or more
polypeptides in the sample, thereby tagging one or more
polypeptides with the reagent; resolving the
polypeptides in the sample; visualizing the one or more
polypeptides tagged with the reagent; contacting the
one or more tagged polypeptides with a capture moiety
for the affinity tag; isolating the one or more tagged
polypeptides; cleaving the linker of the reagent to
release the one or more tagged polypeptides; and
identifying a released tagged polypeptide.

The invention additionally provides a method
of identifying a polypeptide in a sample. The method
can include the steps of contacting a sample with a
VICAT reagent of the invention under conditions
allowing the reactive group to bind to and react with
one or more polypeptides in the sample, thereby
labeling one or more polypeptides with the reagent;
cleaving the polypeptides in the sample to generate
peptide fragments; adding one or more peptide standards
to the sample, wherein the peptide standards correspond
to peptides generated from cleaving sample polypeptides
and are labeled with an isotopically distinct version
of the isotope tag; resolving the labeled peptides in
the sample; visualizing the labeled peptides;
contacting the labeled peptides with a capture moiety
for the affinity tag; isolating the labeled peptides;
cleaving the linker of the reagent to release the
isolated peptides; and identifying a released peptide.
The identifying and/or quantifying step can be
performed using mass spectrometry or tandem mass
spectrometry.

A variety of mass spectrometry systems can be employed in the methods of the invention for identifying and/or quantifying a sample molecule such as a polypeptide. Mass analyzers with high mass accuracy, high sensitivity and high resolution include, but are not limited to, matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometers, electrospray ionization time-of-flight (ESI-TOF) mass spectrometers and Fourier transform ion cyclotron mass analyzers (FT-ICR-MS). Other modes of MS include ion trap and triple quadrupole mass spectrometers. In ion trap MS, analytes are ionized by electrospray ionization or MALDI and then put into an ion trap. Trapped ions can then be separately analyzed by MS upon selective release from the ion trap. Fragments can also be generated in the ion trap and analyzed. Sample molecules such as polypeptides labeled with a VICAT type reagent can be analyzed, for example, by single stage mass spectrometry with a MALDI-TOF or ESI-TOF system. Methods of mass spectrometry analysis are well known to those skilled in the art (see, for example, Yates, _J. Mass Spect._ 33:1-19 (1998); Kinter and Sherman, _Protein Sequencing and Identification Using Tandem Mass Spectrometry_, John Wiley & Sons, New York (2000); Aebersold and Goodlett, _Chem. Rev._ 101:269-295 (2001); Griffin et al., _Curr. Opin. Biotechnol._ 12:607-612 (2001)).

For high resolution polypeptide fragment separation, liquid chromatography ESI-MS/MS or automated LC-MS/MS, which utilizes capillary reverse phase chromatography as the separation method, can be used (Yates et al., _Methods Mol. Biol._ 112:553-569 (1999)). Data dependent collision-induced dissociation (CID) with dynamic exclusion can also be used as the mass spectrometric method(Goodlett, et al., _Anal. Chem._ 72:1112-1118 (2000)).

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Differentially labeled isotope tags can be incorporated into the VICAT reagent (see Example I). The differential tags can be reacted with a test and control sample and run together or in parallel. The differentially labeled molecules can be analyzed using mass spectrometry (MS). Because the sample molecules are differentially labeled with light and heavy isotope tags, the peptide fragments can be distinguished by MS, allowing a side-by-side comparison of the relative amounts of each peptide fragment from a control sample and a test sample. If desired, MS can also be used to sequence the corresponding labeled peptides, allowing identification of molecules corresponding to the tagged peptide fragments.

Moreover, the use of an isotope tag in the VICAT reagent allows the incorporation of various heavy labels into the same molecule. A VICAT reagent having 0, +6 or +12 heavy isotopes is exemplified in Figures 1 and 2. By having chemically identical but differentially isotopically labeled VICAT reagents available, multiple tags can be used in the same experiment. For example, one VICAT reagent can be used to tag sample molecules, a second VICAT reagent can be used to tag a standard molecule for use as an internal standard, and a third VICAT reagent can be used as a marker for the sample molecule in a separation method. Thus, the ability to incorporate various amounts of heavy isotope into an isotope tag provides versatility in the use of the VICAT reagent for labeling and isolating sample molecules, preparing standards, and visualizing sample molecules for isolation and analysis.

The use of multiple isotopic forms of the isotope tag of a VICAT reagent is advantageous for

allowing tagging of a sample molecule, convenient localization of a sample molecule in a separation method, and quantification of the sample molecule by inclusion of an internal standard. As discussed above, a VICAT-labeled standard peptide can be added at relatively high quantities to facilitate localization in a separation method. The sample molecule can be labeled with the same VICAT reagent but with a differential tag, for example, a +6 tag. Furthermore, an internal standard labeled with still another differential tag, for example +12, can be included for quantification. In this case, one VICAT-labeled standard is added at a high concentration for visualization while a second VICAT-labeled standard is added in an amount suitable for quantifying the sample molecule. The internal standard added for quantifying the sample molecule is added in an amount suitable for quantification, that is, within the quantitative range of the detection method. One skilled in the art can readily determine an appropriate amount of internal standard to add to a sample for quantitative analysis and adjust the amount added depending on the type of quantitative analysis used. For example, the amount of internal standard can be adjusted so that the VICAT-labeled sample and standard molecules can be quantified by mass spectrometry. Similarly, one skilled in the art can readily determine a suitable amount of VICAT-labeled standard to use for visualization in a separation method depending on the nature of the detectable moiety on the VICAT reagent and the detection method used.

An advantage of the VICAT method is that a pair of peptides tagged with light and heavy VICAT reagents are chemically identical and therefore serve as mutual internal standards for accurate quantification (Gygi et al., *supra*, 1999). Using MS,

the ratios between the intensities of the lower and
upper mass components of pairs of heavy- and light-
tagged fragments provides an accurate measure of the
relative abundance of the peptide fragments.
Furthermore, a short sequence of contiguous amino
acids, for example, 5-25 residues, contains sufficient
information to identify the unique polypeptide from
which the peptide fragment was derived (Gygi et al.,
supra, 1999). Thus, the VICAT method can be
conveniently used to identify differentially expressed
molecules, if desired.

Furthermore, the methods of the invention are
advantageous in that absolute quantification of a
sample molecule can be determined if a known amount of
VICAT-labeled standard molecule is included as a
standard for quantitative analytical methods. The
inclusion of a detectable tag in a VICAT reagent can be
readily exploited to facilitate quantification and
calibration of a VICAT-labeled standard. If a VICAT
reagent contains a detectable moiety that absorbs
light, the detectable moiety can be used to quantify a
labeled molecule based on the absorption properties of
the detectable moiety. Exemplary detectable moieties
that absorb light include fluorophores such as
coumarin. For example, if the detectable moiety
absorbs in the ultraviolet (UV) range, UV absorbance
can be used to determine the concentration of the
labeled standard peptide. The concentration can be
determined from Beer's law and the known extinction
coefficient of the detectable moiety used in the VICAT
reagent.

As discussed above, an isotope tag is
included in a VICAT reagent, allowing differential
labels to be incorporated into sample and standard
molecules and facilitating quantification of sample

molecules. Thus, a VICAT reagent can be used for two
purposes, localization and quantification. The VICAT
reagent is used to determine the location of a
particular sample molecule based on visualization of
the corresponding VICAT-labeled standard. The VICAT
reagent also allows quantification by MS analysis based
on the incorporation of isotopically distinct isotope
tags, which can be used for quantitative comparison of
the VICAT-labeled sample and standard molecules.

Although generally exemplified herein with
the addition a standard peptide or peptides labeled
with a corresponding isotopically labeled VICAT reagent
for analysis of a particular sample polypeptide, it is
understood that the methods of the invention are also
applicable to the analysis of multiple sample
polypeptides. For example, VICAT-labeled standard
peptides can be added to identify and/or quantify two
different polypeptides in a sample. In such a case, a
particular VICAT reagent can be used to label sample
polypeptides, and the corresponding standard peptides
for the two polypeptides are labeled with an
isotopically distinct version of the same VICAT reagent
as used to label the sample polypeptides. The methods
are advantageous in that multiple polypeptides in a
sample can be analyzed.

Any number of different polypeptides in a
sample can be analyzed so long as there is sufficient
resolution of the standard VICAT-labeled peptides for
further analysis. Thus, the methods of the invention
can be used to identify and/or quantify three or more
polypeptides in a sample, four or more polypeptides,
five or more polypeptides, six or more polypeptides,
seven or more polypeptides, eight or more polypeptides,
nine or more polypeptides, ten or more polypeptides,
twelve or more polypeptides, fifteen or more

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polypeptides, or even grater numbers so long as the
VICAT-labeled peptides can be sufficiently resolved for
further analysis. Corresponding VICAT-labeled
standards for the multiple polypeptides to be analyzed
can be synthesized and added to the sample.

When analyzing multiple polypeptides in a
sample, a single VICAT reagent can be used to label
sample polypeptides. In addition, more than one type
of VICAT reagent can be used to label sample
polypeptides. For example, one type of VICAT reagent
can be used to label reactive sulfhydryl groups of
sample polypeptides and a second type of VICAT reagent
can be used to label reactive amine groups. The use of
VICAT reagents having different reactive groups to
label the same sample can be used to distinguish
between two polypeptides. Furthermore, such VICAT
reagents can also incorporate different detectable
moieties, for example, two different fluorophores or
chromophores. Thus, the use of different types of
VICAT reagents containing different reactive groups
and/or detectable moieties can be used to facilitate
the identification and quantification of multiple
polypeptides in a sample. One skilled in the art can
readily determine suitable VICAT reagents for
differential labeling of two or more polypeptides in a
sample.

The methods of the invention can be used in a
variety of applications. The methods of the invention
are particularly useful for analysis of polypeptides
for which antibodies are not available. For example,
the methods can be used to analyze polypeptides for
which antibodies have not been generated or which are
poorly immunogenic. In the case where new gene
sequences or splice variants are identified, there is
no need to recombinantly express the encoded

polypeptide for antibody production. Instead, only one
or a few peptides that uniquely identify a polypeptide
need to be synthesized, which can be readily predicted
from the nucleic acid sequence.

The methods of the invention can also be
applied to the quantification of specific isoforms of
splice variants. The peptides selected as standards
are chosen based on known or empirically determined
splice variants. The peptide standards are selected
such that, for each alternatively spliced protein,
minimally one peptide is used that is common to all
splice variants and minimally one peptide is used that
is unique for each splice variant tested.

The methods can also be applied to the
detection and quantification of proteins associated
with genetic disease. As such, a genetic disease known
to be associated with aberrant expression of a
polypeptide can be analyzed using methods of the
invention by including a VICAT-labeled peptide standard
corresponding to the aberrantly expressed polypeptide.

An exemplary genetic disease for which
methods of the invention can be applied is muscular
dystrophy (see Example IV). Duchenne and Becker
muscular dystrophies are allelic disorders caused by a
deficiency of the dystrophin protein in muscle (Worton
and Brooke, in The Metabolic and Molecular Bases of
Inherited Diseases, 7th ed., Scriver, ed., ppl 4195-
4112, McGraw-Hill, New York (1994)). The dystrophin
gene, located on the X chromosome at Xp21, is one of
the largest genes in the human genome (2,300 kb),
contains 79 exons, and encodes a 427 kDa cytoskeletal
protein. The dystrophin protein is missing in boys
with Duchenne muscular dystrophy (DMD) and is

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significantly decreased in those with Becker muscular
dystrophy (BMD).

From a diagnostic perspective, approximately
2/3 of affected males can be confirmed to have muscular
dystrophy by molecular methods that can identify
deletions or duplications within the dystrophin gene
(Chamberlain et al., Nucl. Acids Res. 16:11141-11156
(1988)). Confirmatory diagnosis of DMD or BMD in the
remaining 1/3 of patients who have small genetic
alterations typically involves a surgical muscle biopsy
with antibody staining or immunoblot analysis to show
the absence of dystrophin. A truncated dystrophin
revealed by immunoblot analysis, or an in-frame
deletion in the central portion of the gene, would
predict the Becker form of muscular dystrophy. The
average cost associated with an open muscle biopsy and
pathological evaluation exceeds $3000 and places
children with muscular dystrophy at clinical risk from
anesthesia, with the potential for rhabdomyolosis
occurring from the anesthesia.

A VICAT reagent of the invention can be
applied to quantitatively measure dystrophin from very
small muscle samples that can be obtained by needle
biopsy. Such methods are also applicable to other
forms of muscular dystrophy. Thus, the VICAT reagents
of the invention can be used in methods directed to
microtechnology for the quantitative measurement of
specific proteins in muscle tissue. In addition to
diagnostic applications, the methods can also be
applied to measuring the effectiveness of gene therapy
or other therapies used to treat a disease.

The reagents and methods of the invention are
also applicable to diagnosing and determining the
prognosis for various cancers. For example, mRNA

expression array experiments are performed on tumor
samples of various tissues, for example, lung, colon,
liver, ovary, breast, prostate, and the like. The mRNA
expression experiments allow the determination of
tumor-associated changes in gene expression, either up-
regulation or down-regulation, compared to normal
corresponding tissue. Furthermore, it is useful to
determine whether any of the abberantly expressed
proteins, particularly those having increased
expression, are present in blood serum of the patients.
If so, these up-regulated proteins can function as
convenient markers for diagnosis of the corresponding
cancer in serum samples from patients. Exemplary
cancer diagnostic markers include prostate specific
antigen (PSA) for prostate cancer and CA125 for ovarian
cancer. The VICAT reagents and methods of the
invention are particularly useful for rapidly screening
"candidate" diagnostic markers by measuring the level
of the corresponding protein in serum or other bodily
fluids and determining whether the amount of candidate
diagnostic markers correlate with the disease.

       Thus, the invention provides a method of
diagnosing a disease. The method can include the steps
of contacting a sample from a patient with a VICAT
reagent of the invention under conditions allowing the
reactive group to bind to and react with one or more
polypeptides in the sample, thereby labeling one or
more polypeptides with the reagent; cleaving the
polypeptides in the sample to generate peptide
fragments; adding one or more peptide standards to the
sample, wherein the peptide standards correspond to
peptides generated from cleaving sample polypeptides,
are labeled with an isotopically distinct version of
the isotope tag, and are derived from a polypeptide
exhibiting aberrant expression in the disease;
resolving the labeled peptides in the sample;

visualizing the labeled peptides; contacting the
labeled peptides with a capture moiety for the affinity
tag; isolating the labeled peptides; cleaving the
linker of the reagent to release the isolated peptides;
quantifying the released peptides; and comparing the
amount of sample peptide to standard peptide in the
released peptides, wherein the amount of sample peptide
is correlated with the diagnosis of the disease. The
methods can be used to diagnose a variety of diseases
in which aberrant protein expression is associated with
a disease, either increased or decreased expression.

The methods of the invention are particularly
useful for diagnosis of genetic diseases, where one or
a few aberrantly expressed proteins are diagnostic for
a disease. For example, the methods can be used to
diagnose a disease such as muscular dystrophy,
including Duchenne muscular dystrophy or Becker
muscular dystrophy by measuring decreased expression of
dystrophin. Similarly, the methods of the invention
can be used to quantify the expression of prostate
specific antigen (PSA) for diagnosis of prostate
cancer. The methods of the invention can also be used
to identify markers and/or diagnose other cancers such
as lung, colon, liver, ovary, breast, or other cancers.
One skilled in the art will readily recognize that the
methods of the invention are applicable to these and
other diseases associated with aberrant expression of
one or more polypeptides. Furthermore, one skilled in
the art can readily generate corresponding standard
peptides for analysis of the aberrantly expressed
polypeptides using methods of the invention.

Thus, one skilled in the art can readily
apply the methods of the invention to the analysis of
known markers associated with a disease. An increasing
number of protein markers for a variety of diseases are

becoming available through genomics and proteomics
analysis. For example, mRNA expression arrays can be
used to determine differential expression between a
healthy individual and an individual having a disease.
VICAT-labeled standards corresponding to the
differentially expressed mRNAs can be synthesized and
used for qualitative and/or quantitative diagnostic
analysis of patient samples of the same tissue.
Furthermore, if the differentially expressed mRNAs
encode one or more secreted proteins or peptides, the
methods of the invention can be applied to blood or
serum samples to analyze differentially expressed
secreted proteins correlated with a disease state.

The methods of the invention are also
applicable to the detection and quantification of
patterns of protein expression that are diagnostic or
prognostic for a disease. The methods can be used in
diagnostic applications in body fluids such as blood,
serum or plasma, cerebrospinal fluid, urine, saliva,
seminal plasma, pancreatic juice, breast milk, lung
lavage, and the like. Because the methods can be used
to analyze and quantify multiple peptides in the same
analysis, the methods are particularly useful for
diagnostic applications since the ability to analyze
multiple polypeptides in the same analysis allows
multiple peptides correlated with a diagnostic
application to be used rather than the analysis of one
diagnostic marker alone. Thus, the methods can be
applied to the analysis of multiple diagnostic markers
associated with a disease. Once a set of diagnostic
markers has been identified, the methods of the
invention can be applied to quantify the set of markers
for an analysis that is expected to be more informative
than the analysis of a single diagnostic marker alone.

Diagnostic patterns of protein expression can
be determined by direct comparative, quantitative
protein profiling of tissues or body fluids of persons
afflicted with a particular disease and healthy control
individuals (Gygi et al., Nat. Biotechnol. 17:994-999
(1999); WO 00/11208). Alternatively, diagnostic
protein expression patterns can be determined from gene
expression array measurements in afflicted tissue of an
individual having a disease and healthy control tissue
of the same type. In addition, specific marker
proteins can be identified from known disease markers
found in the scientific literature. One skilled in the
art will recognize that these and other methods for
determining or identifying differential protein
expression correlated with a disease state can be used
to identify protein disease markers suitable for
diagnostic applications using VICAT reagents and
methods of the invention.

The invention additionally provides a kit
containing one or more VICAT reagents. For example,
the kit can contain a single VICAT reagent useful for
labeling and identifying a polypeptide. In addition, a
kit can contain a set of two or more differentially
isotopically labeled VICAT reagents, which is
particularly useful for quantitative analysis using
mass spectrometry. The contents of the kit of the
invention, for example, one or more VICAT reagents, are
contained in packaging material, and, if desired, a
sterile, contaminant-free environment. In addition,
the packaging material contains instructions indicating
how the materials within the kit can be employed to
label sample molecules. The instructions for use
typically include a tangible expression describing the
reagent concentration or at least one assay method
parameter, such as the relative amounts of reagent and
sample to be admixed, maintenance time periods for

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reagent/sample admixtures, temperature, buffer
conditions, and the like.

It is understood that modifications which do
not substantially affect the activity of the various
embodiments of this invention are also provided within
the definition of the invention provided herein.
Accordingly, the following examples are intended to
illustrate but not limit the present invention.

EXAMPLE I
Chemical Structure and Synthesis of VICAT Reagents

This example describes a synthetic scheme for
VICAT reagents.

The structure of exemplary VICAT reagents and
their corresponding synthetic schemes are shown in
Figures 1, 2, 4, 5 and 6. A representative reagent
contains the biotin and iodoacetyl features that are
present in the ICAT reagents (Gygi et al., Nature
Biotechnol. 17:994-999 (1999) and also contains a
tetramethylrhodamine to make the reagent highly colored
and fluorescent. The iodoacetyl group is attached to
an ethylenediamine unit, which is attached to the rest
of the VICAT reagent via a photolabile 2-
nitrobenzyloxycarbonyl group. Exposure of the VICAT-
peptide conjugate to long wavelength UV light (using a
handheld lamp) for several minutes leads to
photocleavage of the bond between the benzyl carbon and
the bridging ester oxygen, yielding Peptide-S-
$CH_2CONHCH_2CH_2NH-COOH$. The latter undergoes spontaneous
decarboxylation (Olejnik et al., Proc. Natl. Acad. Sci.
USA 92:7590-7594 (1995) to yield Peptide-S-
$CH_2CONHCH_2CH_2NH_2$. This peptide cysteine-bound N-
aminoethylacetamido group is abbreviated NAMETAG.

Also shown in Figures 1 and 2 are the VICAT(+6) and VICAT(+12) reagents, which contain heavy isotopes in the NAMETAG portion, thus increasing their mass by +6 and +12, respectively. After photocleavage, the NAMETAG group, with or without isotopic substitution, is left on the peptide so that the peptides can be quantified by the ICAT-based method (Gygi et al., *supra*, 1999).

Three different peptides, 10-15 amino acids in length and containing a single NAMETAG-derivatized cysteine, were found to undergo normal peptide bond fragmentation during tandem ESI-MS. Thus, the presence of the NAMETAG does not interfere with obtaining peptide sequence information. Additionally, the amide in the NAMETAG moiety eliminates ethylenediamine upon collision-induced dissociation (CID), which serves to fingerprint by MS/MS those peptides that contain the NAMETAG. The VICAT reagents also have the advantageous property of being water soluble at the concentrations needed for peptide mixture analysis. In particular, the presence of the tertiary amine in the linker will be protonated at pH near neutrality or lower and thus contribute to reagent solubility in water.

The NBD-VICAT reagent shown in Figure 1A was synthesized as shown in Figure 1B. Briefly, tetrahydrofuran (THF) was distilled from sodium metal/ benzophenone ketyl. Methylene Chloride (CH_2Cl_2), and triethylamine (TEA) were distilled from CaH_2. Anhydrous dimethylformamide (DMF) and pyridine were purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade solvents were purchase from Fisher (Fair Lawn, NJ). Water for HPLC was purified at point-of-use by a Milli-Q water system (Millipore, Bedford, MA). Trifluoroacetic acid (TFA) was purchased from Advanced ChemTech (Louisville, KY), (+)-Biotin, di-t-butyl

dicarbonate, diethylenetriamine, ethylenediamine, and iodoacetic anhydride are from Aldrich Chemical Co. (Milwaukee, WI), and 4-Fluoro-7-nitrobenzofurazan (NBD-F) is from Dojindo Molecular Technologies, Inc. (Gaithersburg, MD). All other reagents were used as received. Unless otherwise noted, all non-aqueous reactions were carried out under Argon atmosphere with oven-dried glassware.

For spectral analyses, NMR spectra were recorded on Bruker spectrometers: AC-200 (200 MHz), AC-300 (300 MHz) or AC-500 (500MHz). In rare cases, NMR data had to be omitted for proteins that are likely to be buried under the NMR peak from the solvent. Mass spectral data was obtained using Bruker Daltonics (Bremen, Germany) Esquire electrospray-ion trap mass spectrometer.

For chromatography, silica gel flash chromatography was performed using 230-400 mesh silica gel 60 (Merck, Darmstadt, Germany). Thin-Layer chromatography was performed on silica 60 with F254, aluminum-backed plates (Merck). Reversed-phase HPLC separations were performed using a Vydac (Hisperia, CA) semi-preparative column (up to 5 mg total loading, 10 μm packing, 10 mm x 250 mm, catalog no. 218TP1010) at 4 mL/min, or a preparative column (up to 300 mg total loading, 10 μm packing, 20mm x 250 mm, catalog no 218TP1022) at 6 mL/min.

Compound 1. (+)-Biotin (3g, 12.3 mmol) was dissolved in warm anhydrous DMF (60 mL). After the reaction was cooled to room temperature, TEA (3.2 mL, 24.6 mmol) was added, followed by tetrafluorophenyl trifluoroacetate (4.85g, 18.5 mmol, prepared according to the literature). After being stirred for 30 minutes at room temperature, the solvent was removed under reduced

pressure. The residue was crystallized with diethyl ether, and the product filtered (compound 1). The solid was dried under vacuum to afford 3.9 g (81%) of a white solid. ^{1}H-NMR (300 MHz, DMSO-d^6) δ 7.9 (m, 1H); 6.4 (2H, d); 4.2 (2H, dt); 3.2 (m, 1H); 2.75-2.9 (m, 3H), 2.6 (1H, d); 1.4-1.8 (m, 6H).

Compound 2. To a round bottom flask containing diethylenetriamine (5.5 g, 51 mmol) at 0°C was transferred via cannula a solution of compound 1 (2g, 5.10 mmol) in DMF (40 mL), also at 0°C. After being stirred for 30 min at 0°C, solvent was removed under reduced pressure. The resulting residue was crystallized with ethyl ether (100 mL), filtered, and washed with acetone (50 mL). The solid was left under vacuum overnight, and 1.2 g (71%) of the desired product was obtained (compound 2). ^{1}H-NMR (300 MHz, DMSO-d^6) δ 6.4 (2H, d); 4.2 (2H, dt); 3.2 (m, 3H); 3.0-2.4 (m, 8H); 2.1 (t, 2H), 1.2-1.7 (m, 6H). ESI-MS (M+H$^+$)$^+$: 330.4, (M+2H$^+$)$^{2+}$: 659.4.

Compound 3. To a round bottom flask containing a solution of compound 2 (300 mg, 0.91 mmol) in DMF (30 mL) at 0°C was slowly added a solution of di-t-butyl dicarbonate (200 mg, 0.91 mmol) in DMF (2 mL). After being stirred for an hour at 0°C, the ice bath was removed, and the reaction was left stirring overnight at room temperature. The solvents were concentrated under reduced pressure without heating. A mixture of ethyl ether and methylene chloride was added to the residue, and the resulting solution was stirred for 3-4 h. The final product was filtered, and dried under vacuum to afford a white solid (compound 3) (290 mg, 74.4%). ^{1}H-NMR (300 MHz, DMSO-d^6) δ 6.4 (2H, d); 4.2 (2H, dt); 3.2 (3H, m); 3.0-2.4 (m, 8H); 2.1 (m, 2H), 1.2-1.7 (m, 15H). ESI-MS (M+H$^+$)$^+$: 430.5.

Compound 4. To a vial containing a solution of
compound 3 (130 mg, 0.30 mmol) in DMF (3 mL) was added
a solution of 5-bromomethyl-1-(1-hydroxyethyl)-2-
nitrobenzene (prepared according to the literature
method, the material used also contained 1 part in 4.6
of 5-methyl-1-(1-hydroxyethyl)-2-nitrobenzene) (160 mg)
in DMF (1 mL), followed by TEA (86 μL, 0.62 mmol). The
reaction mixture was stirred at room temperature for 7
days, and then placed in a shaker at 37°C for 2 days.
The solvents were removed in a speed-vacuum apparatus,
and the reaction mixture was injected onto the HPLC
column. Preparative C_{18} column, detector λ= 240nm, A=
H_2O with 0.08% TFA, B= acetonitrile (MeCN) with 0.08%
TFA, gradient: 0'-20': 20% B; 20'-80': 100% B. Product
(compound 4) (50 mg, 27%) came out at 44% B. ^{1}H-NMR
(300 MHz, MeOH-d^4) δ 8.05 (1H, s); 8.0 (1H, d); 7.7
(1H, d); 5.35 (1H, q); 4.5 (1H, dd); 4.25 (1H, dd); 3.6
(2H, bs); 3.45-3.25 (m, 8H); 3.2 (m, 1H), 2.95(1H, dd);
2.7(1H, d); 2.2 (2H, t); 1.8-1.25 (m, 6H); 1.5 (3H, d);
1.4 (9H, s). ESI-MS (M+H$^+$)$^+$: 609.5, (M+ Na$^+$)$^+$: 631.5.

Compound 5. To a vial containing a solution of
compound 4 (70 mg, 0.12 mmol) in a 1:1.75 mixture of
DMF: CH_2Cl_2 (total volume of 550 μL) was added a
solution of carbonyl diimidazole (28 mg, 0.17 mmol) in
CH_2Cl_2 (850 μL). After being stirred at room
temperature for 2 h in the presence of 4 angstrom
molecular sieves, ethylenediamine (32 μL, 0.23 mmol)
was added. After an additional 1.5 h, the solvent was
removed in a speed-vacuum apparatus. The residue was
injected onto the HPLC column. Preparative C_{18} column,
λ= 240nm, A= H_2O with 0.08% TFA, B= MeCN with 0.08%
TFA, gradient: 0'-20': 20% B; 20'-80': 100% B. Product
(compound 5) as TFA salt (43.5mg, 47%) came out at 40%
B. First intermediate: ESI-MS (M+H$^+$)$^+$: 703.6, (M+
Na$^+$)$^+$: 725.6; Final product 5: ESI-MS (M+H$^+$)$^+$: 695.7,
(M+ Na$^+$)$^+$: 717.7.

Compound 6. To a vial containing compound 5 (43.5 mg, 0.054 mmol) in THF (1 mL) in the presence of 4 angstrom molecular sieves, was added TEA (6 µL, 0.054 mmol), followed by a solution of iodoacetic anhydride (21 mg, 0.059 mmol) in THF (1 mL). After stirring for 1.5 h at room temperature, an excess of 1 equivalent of TEA and 0.5 equivalents of iodoacetic anhydride were added to the reaction mixture. After an additional 1.5 h at room temperature, the solvents were removed in a speed-vacuum apparatus. The residue was injected onto the HPLC column. Preparative C_{18} column, λ= 240nm, A= H_2O with 0.08% TFA, B= MeCN with 0.08% TFA, gradient: 0'–20': 20% B; 20'-80': 100% B. Product (23.4 mg, 55%) came out at 49% B (compound 6). ESI-MS $(M+H^+)^+$: 863.7, $(M+ Na^+)^+$: 885.6

Compound 7. To a vial containing a solution of compound 6 (23.4 mg, 0.027 mmol) in $CHCl_3$ (0.4 mL) was added TFA (80 µL). The reaction mixture was stirred for 1.5 h at room temperature. Solvent was removed using a speed-vacuum apparatus. Methanol (MeOH) was added, and the resulting solution was concentrated in the speed- vacuum apparatus. This procedure was repeated 3 more times to remove excess TFA. The TFA salt of the product (compound 7) was obtained in 98% yield (23.4 mg) as oil. ESI-MS $(M+H^+)^+$: 763.5.

Compound 8. To a vial containing compound 7 (10 mg, 0.011 mmol) was added a solution of NBD-F (7.2 mg, 0.027 mmol) in DMF (640 µL), followed by Hunig's base (diisopropylethylamine) (8 µL, 0.046 mmol). After being stirred at room temperature for 1 hr in the presence of 4 angstrom molecular sieves, the solvents were removed in a speed-vacuum apparatus. The residue was injected onto the HPLC column. Preparative C^{18} column, λ= 500nm, A= H_2O, B= MeCN, gradient: 0'-20':

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20% B; 20'-80': 100% B. An orange solid (compound
8) (4.6 mg, 43.8%) came out at 51% B. ^{1}H-NMR (500 MHz,
MeOH-d^4) δ 8.43 (1H, d); 7.6 (2H, bs); 7.45 (1H, d);
6.16 (1H, bs); 6.05 (1H, q); 36.8 (2H, bs); 3.65 (2H,
m); 3.25-3.15 (m, 6H); 2.96-2.88 (6H, m); 2.8 (1H, t);
2.71 (1H, d); 2.22 (2H, t); 1.8-1.55 (4H, m); 1.5 (3H,
d); 1.42 (12H, m). ESI-MS (M+H$^+$)$^+$: 926.6, (M+ Na$^+$)$^+$:
948.7.

Additional exemplary VICAT reagents can be
readily synthesized by the route shown in Figure 4B.
The VICAT reagent shown in Figure 4 is an exemplary
VICAT reagent having a radioactive detectable moiety.
The ^{14}C-acetyl-VICAT reagent is synthesized (see Figure
4B) in a manner substantially similar to that described
above for other VICAT reagents. Briefly, compound 1 is
reacted with ethylenediamine in DMF, followed by
reaction with 5-bromomethyl-1-(1-hydroxyethyl)-2-
nitrobenzene in DMF to generate compound 2. Compound 2
is reacted with di-t-butyl dicarbonate in DMF. To the
product of this reaction is added carbonyldiimidazole
in DMF, followed by ethylenediamine. To the product of
this reaction is added iodoacetic anhydride, followed
by TFA, to generate compound 3. Compound 3 is
dissolved in DMF, and sodium 1-[^{14}C]acetate, TEA, and
BOP (Benzotriazol-1-yloxytris(dimethylamino)phosphonium
hexafluorophosphate) are added to generate compound 4
(^{14}C-acetyl-VICAT).

The isotopically labeled VICAT reagents are
prepared as follows (Figures 1 and 2 insert). Di-^{13}C-
labeled oxalic acid (Cambridge Isotopes; Andover MA) is
distilled from ethanol/H$_2$SO$_4$ to give diethyl oxalate,
which is treated with NH$_4$OH in ethanol to give di-^{13}C-
labeled oxamate, (^{13}CONH$_2$)$_2$ (Wilmes and Winnewisser, _J._
Labelled Compd. Radiopharm. 31:1037-1040 (1992). The
latter is reduced with LiAlD$_4$ (Aldrich) in

tetrahydrofuran (THF) (Thomas, Deuterium Labeling in
Organic Chemistry, Appleton-Century-Crofts, New York
(1971)) to give $H_2N^{13}CD_2{}^{13}CD_2NH_2$. Repetition of this
sequence of chemical reactions using $^{15}NH_4OH$ (Cambridge
Isotopes)(excess $^{15}NH_3$ can be fully recovered by bulb-
to-bulb distillation) gives $H_2{}^{15}N^{13}CD_2{}^{13}CD_2{}^{15}NH_2$.

The labeled ethylenediamines is reacted with
di-t-butyldicarbonate to give the mono-BOC derivatives
(Lochner et al., Helv. Chim. Acta 81:2270-2281 (1998)).
Di-^{13}C-labeled acetic acid (Cambridge Isotopes) is
heated in D_2O/K_2CO_3 for 3-5 h at 120°C (Atkinson et al.,
J. Am. Chem. Soc. 90: 498-499 (1968)) to give
$^{13}CD_3{}^{13}COOD$, which is reacted with phosphorus and Br_2 to
give $Br^{13}CD_2{}^{13}COBr$ (Ward, J. Chem. Soc. 1160-1165
(1922)). The labeled bromoacetyl bromide is reacted
with the BOC-protected ethylenediamine, followed by BOC
removal with trifluoroacetic acid, to give
$Br^{13}CD_2{}^{13}CO^{15}NH^{13}CD_2{}^{13}CD_2{}^{15}NH_2$, which is treated with KI in
acetone to give the desired $I^{13}CD_2{}^{13}CO^{15}NH^{13}CD_2{}^{13}CD_2{}^{15}NH_2$
(12 atomic mass units (amu) higher than the non-
isotopically substituted compound). Likewise, the +6
amu shifted compound $ICH_2CONH^{13}CD_2{}^{13}CD_2NH_2$ is prepared in
the same way. All of these reactions proceed in near
quantitative yield and make use of relatively
inexpensive heavy isotope labeled materials. The
conversion of $H_2NCH_2CH_2NH_2$ to $ICH_2CONHCH_2CH_2NH_2$ is
obtained in 80% overall yield with non-labeled
materials.

Synthetic schemes of additional exemplary VICAT
reagents are described below. For preparation of the
VICAT reagents, tetrahydrofuran (THF) was distilled from
sodium/benzophenone under Ar, and methylene chloride
(CH_2Cl_2), and triethylamine (TEA) were distilled from CaH2
under Ar. Anhydrous dimethylformamide (DMF) and pyridine

were purchased from Aldrich. [^{13}C, ^{15}N]-potassium cyanide,
1,2-[^{13}C$_2$]-dibromoethane from Aldrich. All other compounds
are reagent graded and used as received. Unless otherwise
noted, all non-aqueous reactions were carried out under
Ar with oven-dried glassware. Mass spectral data was
obtained using a Bruker Daltonics Esquire electrospray-
ion trap mass spectrometer. Silica gel flash
chromatography was performed using 230-400 mesh silica
gel 60 (Merck, Darmstadt, Germany). Thin-Layer
chromatography was performed on silica 60 with F254
indicator, aluminum-backed plates (Merck). Reverse-phase
HPLC separations were performed using a Vydac (Hisperia,
CA) semi-preparative column (up to 5 mg total loading, 10
μm packing, 10 mm x 250 mm, catalog no 218TP1010) at 4
mL/min, or a preparative column (up to 300 mg total
loading, 10 μm packing, 20 mm x 250 mm, catalog no
218TP1022), or an a large preparative column (up to 1 g
total loading, 10-15 μm packing, 5 x 25 cm, catalog no
218TP101550).

        For synthesis of VICAT$_{SH}$ reagents, the following
synthetic scheme was used. A portion of the synthetic
scheme is shown in Figure 5.

Compound 9. (+)-Biotin (4.5 g, 183 mmol) was dissolved in
warm anhydrous DMF (90 mL). After cooling to room
temperature, TEA (4.7 mL, 36.7 mmol) was added, followed
by dropwise addition of tetrafluorophenyl
trifluoroacetate (7.3 g, 27.4 mmol, (Gamper et al., Nucl.
Acids Res. 21:145-150 (1993)). After being stirred for
30 minutes at room temperature, the solvent was removed
under reduced pressure. The residue was crystallized
with dry diethyl ether, and the product filtered. The

solid was dried under vacuum to afford 6.2 g (86%) of a white solid. ^{1}H-NMR (300 MHz, d$_6$-DMSO) δ 7.9 (m, 1H); 6.4 (2H, d); 4.2 (2H, dt); 3.2 (m, 1H); 2.75-2.9 (m, 3H), 2.6 (1H, d); 1.4-1.8 (m, 6H).

Compound 10. To a round bottom flask containing ethylenediamine (8.5 g, 141.7 mmol) at 0°C was transferred via cannula a solution of compound 9 (5 g, 12.8 mmol) in dry DMF (100 mL), also at 0°C. After stirring for 120 minutes at 0°C, ethyl ether (100 mL) was added and the solid filtered and washed with ether. The white solid was left under vacuum overnight, and 3.15 g (86 %) of the product was obtained. ^{1}H-NMR (300 MHz, d$_6$-DMSO) δ 7.75 (bs, 1H); 6.4 (d, 2H); 4.35 (t, 1H); 4.15 (t, 1H); 3.15-3.0 (m, 3H); 2.85 (dd, 1H); 2.6 (m, 3H); 2.1 (t, 2H), 1.2-1.7 (m, 6H). ESI-MS (M+H$^+$)$^+$: 287.0, (2M+H$^+$)$^+$: 573.0.

Compound 11. To a round bottom flask containing a solution of compound 10 (2.4 g, 8.4 mmol) in dry DMF (72 mL) and diisoproylethylamine (DIPEA) (2.9 mL, 16.7 mmol) at 0°C was slowly added a solution of 5-bromomethyl-2-nitro-acetophenone (Doppler et al., <u>Helv. Chim. Acta</u> 62:271 (1979); Senter et al., <u>Photochem. Photobiol.</u> 42:231-237 (1988)) (2.4 g, 9.3 mmol) in dry DMF (24 mL). After stirring for 3 h at 0°C, the pH was adjusted to ~5 with concentrated HCl (moist pH paper), and the solvent was removed *in vacuo*. The product was purified on a silica column packed with 5:1 CH$_2$Cl$_2$:methanol. The column was run with the same solvent, and the product was obtained in 64 % yield (2.5 g). ^{1}H-NMR (300 MHz, d$_4$-MeOH) δ 8.0 (d, 1H, J= 8.3 Hz); 7.75 (m, 2H); 4.45 (dd, 1H); 4.25 (dd, 1H); 4.1 (s, 2H); 3.4 (t, 2H); 3.2 (m, 1H);

2.95 (m, 3H); 2.85 (d, 1H); 2.55 (s, 3H); 2.1 (t, 2H), 1.25-1.8 (m, 6H). ESI-MS $(M+H^+)^+$: 464.2, $(M+Na^+)^+$: 486.1.

Compound 12. To a round bottom flask containing a solution of compound 11 (2.5 g, 5.4 mmol) in MeOH (100 mL) and TEA (750 µL, 5.4 mmol) was added di-t-butyl dicarbonate (1.3 g, 5.9 mmol). The mixture was stirred at 50°C for 2 h and then cooled to room temperature. Sodium borohydride (650 mg, 16.2 mmol) was added, and the reaction was stirred for an additional hour. Solvent was removed under reduced pressure and the residue resolubilized in CH_2Cl_2 (80 mL). The resulting solution was washed with water (40 mL), brine (40 mL), and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure to afford the product in 78 % overall yield (2.4 g). ^{1}H-NMR (300 MHz, CDCl$_3$) δ 7.8 (d, 1H, J= 8.4 Hz); 7.75 (bs, 1H); 7.2 (m, 1H); 6.9 (d, 1H); 5.9 (d, 1H); 5.35 (m, 1H); 4.4 (m, 3H); 4.25 (m, 1H); 4.1 (d, 1H); 3.0-3.4 (m, 5H); 2.8 (dd, 1H); 2.65 (d, 1H); 2.1 (m, 2H); 1.8 (bs, 1H), 1.20-1.75 (m, 18H). ESI-MS $(M+Na^+)^+$: 588.5.

Compound 13. To a round bottom flask containing a solution of compound 12 (224 mg, 0.4 mmol) in dry CH_2Cl_2 (4 mL) was added a solution of carbonyl diimidazole (97.2 mg, 0.6mmol) in dry CH_2Cl_2 (1 mL). After 2 h stirring at room temperature in the presence of 4 Å molecular sieves, ethylenediamine (53.4 µL, 0.8 mmol) was added. After 1 h, the solvent was removed in a Speed-Vac (Savant Instruments). The residue was purified by HPLC: preparative C_{18} column, λ= 240 nm, 6 mL/min, solvent A = H_2O with 0.08% trifluoroacetic acid (TFA), solvent B = CH_3CN with 0.08% TFA, gradient: 0'-20': 20% B; 20'-70': 70% B. The TFA salt of the product (244 mg, 79.7%) eluted

between 38.7 and 44.3% B. ^{1}H-NMR (300 MHz, d4-MeOH-) δ 7.9
(d, 1H); 7.5 (m, 1H); 7.35 (dd, 1H); 6.2 (m, 1H); 4.6
(bd, 2H); 4.45 (dd, 1H); 4.3 (m, 1H); 3.25-3.5 (m, 6H);
3.2 (m, 1H); 3.0 (m, 2H); 2.8 (dt, 1H); 2.65 (dd, 1H);
2.2 (m, 2H); 1.8 (bs, 1H), 1.25-1.8 (m, 18H). ESI-MS
(M+H$^+$)$^+$: 652.4, (M+ Na$^+$)$^+$: 674.3.

Compound 14. To a vial containing compound 13 (244 mg,
0.32 mmol) in dry THF (8 mL) in the presence of 4 Å
molecular sieves, was added diisoproylethyl amine (DIPEA)
(112 µL, 0.64 mmol), followed by a solution of iodoacetic
anhydride (170 mg, 0.48 mmol) in dry THF (2 mL). After
1.5 h stirring at room temperature, the pH was adjusted
to ~5 with TFA (moist pH paper). The solvent was removed
under reduced pressure and the residue purified by HPLC.
Preparative C$_{18}$ column, λ = 240 nm, 6 mL/min, gradient:
0'-20': 20% B; 20'-80': 70% B. The product (160 mg, 61%)
eluted between 50.5-52% B. ^{1}H-NMR (300 MHz, d$_4$-MeOH) δ
7.95 (d, 1H); 7.55 (m, 1H); 7.35 (dd, 1H); 6.2 (m, 1H);
4.55 (bs, 2H); 4.45 (dd, 1H); 4.25 (dd,1H); 3.65 (bs,
2H); 3.1-3.45 (m, 9H); 2.9 (dd, 1H); 2.65 (dd, 1H); 2.2
(m, 2H); 1.25-1.8 (m, 18H). ESI-MS (M+ Na+)+: 843.2

Compound 15. To a vial containing a solution of compound
14 (160 mg, 0.195 mmol) in CHCl$_3$ (0.5 mL) was added TFA
(200 µL). The reaction mixture was stirred for 1.5 h at
room temperature. Solvent was removed in a Speed-Vac.
MeOH was added, and the resulting solution was
concentrated in a Speed-Vac. This procedure was repeated
3 times to remove excess TFA. The TFA salt of the
product was obtained in 98.5% yield (160 mg). ^{1}H-NMR (300
MHz, d$_4$-MeOH) δ 8.0 (d, 1H); 7.8 (bs, 1H); 7.6 (dd, 1H);

6.1 (m, 1H); 4.5 (m, 1H); 4.4 (bd, 2H); 4.3 (dd, 1H); 4.25 (dd, 1H); 3.7 (bs, 2H); 3.55 (m, 2H); 3.0-3.45 (m, 6H); 2.9 (dd, 1H); 2.65 (dd, 1H); 2.2 (t, 2H); 1.25-1.8 (m, 9H). ESI-MS $(M+H^+)^+$: 720.

^{14}C-VICAT$_{SH}$(-28). To an eppendorf tube containing a solution of compound 15 (7.34 mg; 8.8 μmol) in MeOH (272 μL), was added 1% [^{14}C] formaldehyde in water (v/v, 51 μL, 17.6 μmol, 1 mCi, specific activity 56 Ci/mole, Perkin-Elmer) followed by 1.2 mM methanolic NaCNBH$_3$ (15 μL, 17.6 μmol) and 1.2 mM methanolic DIPEA (22 μL, 26.4 μmol). The closed eppendorf was placed in a shaker at 168 rpm for 6 h. The pH was adjusted to ~5 (moist pH paper) with a 1.2 mM aqueous solution of TFA (20 μL). The mixture was diluted with 30% CH$_3$CN:water, and injected on to the HPLC column. Preparative C$_{18}$ column, λ = 240 nm, 6 mL/min, gradient: 0'-20': 20% B; 20'-80': 70% B. The product (4.6 mg, 0.19mCi) eluted between 34.4-36.7% B.

Compound 16. To a round bottom flask containing a solution of compound 12 (2.0 g, 3.54 mmol) in dry CH$_2$Cl$_2$ (20 mL) was added a solution of carbonyl diimidazole (860 mg, 5.31 mmol) in dry CH$_2$Cl$_2$ (20 mL). After 2 h stirring at room temperature in the presence of 4 Å molecular sieves, putrecine (623 mg, 7.08 mmol) was added. After 2 additional hours, the solvent was removed in a Speed-Vac. The residue was solubilized in water and the pH adjusted to ~5 with TFA. The solution was injected onto the HPLC column. Large preparative C$_{18}$ column, λ = 240 nm, 50 mL/min, gradient: 0'-10': 20% B; 10'-60': 70% B. The product as the TFA salt (2.15 g, 77 %) eluted between 37 and 41.7 % B. ^{1}H-NMR (300 MHz, d$_4$-MeOH-d^4) δ 7.9 (d, 1H);

7.5 (m, 1H); 7.35 (dd, 1H); 6.2 (m, 1H); 4.6 (bd, 2H);
4.45 (dd, 1H); 4.3 (m,1H); 3.25-3.5 (m, 4H); 3.2 (m,
1H); 3.1 (m, 2H); 2.95 (m, 3H); 2.8 (dd, 1H); 2.2 (m,
2H); 1.25-1.8 (m, 22H). ESI-MS (M+H+)+: 680.5.

Compound 17. To a vial containing compound 16 (1.0 g,
1.26 mmol) in dry THF (15 mL) in the presence of 4 Å
molecular sieves, was added DIPEA (440 µL, 2.52 mmol),
followed by a solution of iodoacetic anhydride (670 mg,
1.89 mmol) in dry THF (5 mL). After 1.5 h stirring at
room temperature, the pH was adjusted to ~5 with TFA
(moist pH paper). The solvents were removed under
reduced pressure, and the residue purified by HPLC.
Large preparative C_{18} column, λ = 240 nm, 50 mL/min,
gradient: 0'-10': 20% B; 10'-80': 70% B. The product (657
mg, 61 %) eluted at 46.6 % B. 1H-NMR (300 MHz, d_4-MeOH) d
8.0 (d, 1H, J= 8.3 Hz); 7.6 (m, 1H); 7.4 (d, 1H, J= 8.3
Hz); 6.15 (m, 1H); 4.55 (d, 2H); 4.5 (dd, 1H); 4.3 (dd,
1H); 3.65 (bs, 2H); 3.1-3.45 (m, 9H); 2.95 (dd, 1H); 2.75
(dd, 1H); 2.2 (t, 2H); 1.25-1.8 (m, 22H). ESI-MS $(M+H^+)^+$:
848.0; $(M+Na^+)^+$: 870.5.

Compound 18. To a vial containing a solution of compound
17 (603 mg, 0.71 mmol) in $CHCl_3$ (3 mL) was added TFA (1.2
mL). The reaction mixture was stirred for 2 h at room
temperature. Solvent was removed using in a Speed-Vac.
MeOH was added, and the resulting solution was
concentrated in the Speed-Vac. This procedure was
repeated 3 times to remove excess TFA. The TFA salt of
the product was obtained in 100 % yield (611 mg). ^{1}H-NMR
(300 MHz, d_4-MeOH) δ 8.0 (d, 1H); 7.8 (bs, 1H); 7.6 (dd,
1H); 6.1 (m, 1H); 4.5 (m, 1H); 4.4 (bd, 2H); 4.3 (dd,

1H); 4.25 (dd, 1H); 3.65 (bs, 2H); 3.55 (m, 2H); 3.1-3.35 (m, 7H); 2.95 (dd, 1H); 2.65 (dd, 1H); 2.25 (t, 2H); 1.4-1.8 (m, 13H). ESI-MS (M+H⁺)⁺: 748.4.

^{14}C-VICAT$_{SH}$. To an eppendorf tube containing a solution of compound 18 (7.54 mg; 8.8 μmol) in MeOH (272 μL), was added 1% [^{14}C]formaldehyde in water (v/v, 59 μL, 17.6 μmol, 1 mCi, 49.5 Ci/mole) followed by 1.2 mM methanolic NaCNBH$_4$ (15 μL, 17.6 μmol) and 1.2 mM methanolic DIPEA (22 μL, 26.4 μmol). The capped eppendorf was placed in a shaker at 168 rpm for 6 h. The pH was adjusted to ~5 (moist pH paper) with a 1.2 mM aqueous solution of TFA (20 μL). The reaction was diluted with 30% CH$_3$CN:water, and injected on to the HPLC column. Preparative C$_{18}$ column, λ = 240 nm, 6 mL/min, gradient: 0'-10': 20% B; 10'-70': 70% B. The product (3.5 mg, 0.162 mCi) eluted between 36.8-39.3% B.

VICAT$_{SH}$. The non-radioactive reaction was carried out as for ^{14}C-VICAT$_{SH}$ using compound 18 (1.09 g, 1.27 mmol), 37% formaldehyde in water (207 μL, 2.54 mmol), NaCNBH$_3$ (168 mg, 2.54 mmol), MeOH (50 mL) and DIPEA (662 μL, 3.81 mmol). The product VICAT$_{SH}$ (900 mg, 82%) eluted at 31.2% B. ^{1}H-NMR (300 MHz, d$_4$-MeOH) δ 8.0 (d, 1H); 7.8 (s, 1H); 7.6 (dd, 1H); 6.1 (m, 1H); 4.5 (dd, 1H); 4.25 (dd, 1H); 3.65 (bs, 2H); 3.6 (m, 2H); 3.1-3.45 (m, 8H); 2.95 (m, 4H); 2.65 (dd, 1H); 2.25 (t, 2H); 1.25-1.8 (m, 13H). ESI-MS (M+H⁺)⁺: 762.3.

Compound 19. Potassium [^{13}C, ^{15}N]cyanide (176.3 mg, 2.63 mmol) and [1,2-^{13}C$_2$]-dibromoethane (0.5g, 2.63 mmol) were refluxed for 5 h in 70% ethanol (2 mL). The reaction

mixture was cooled to room temperature and passed through a silica plug with benzene. The solvents were removed under reduced pressure to afford the product in 80% yield (90 mg). ^{1}H-NMR (300 MHz, CDCl$_3$) δ 3.0 (m, 2H); 2.55 (m, 2H).

Compound 20. Compound 19 (137.1 mg, 1.59 mmol) and a 1 M solution of borane in dry THF (16 mL, 15.9 mmol) were refluxed for 24 h. After the reaction was cooled to room temperature, anhydrous ethanol (18 mL) was added. The reaction was stirred for 24 h, and HCl gas was bubbled through the solution for 10 minutes, or until saturation. The di-chloride salt was filtered, washed with anhydrous ethanol, and dried under vacuum to afford 146.5 mg (55.2%) of a white solid. ^{1}H-NMR (300 MHz, D$_2$O) δ 3.5 (m, 2H); 2.75 (m, 2H); 2.15 (m, 2H); 1.75 (m, 2H).

Compound 21. A solution of compound 20 (30 mg, 0.18 mmol), and TEA (368 µL, 2.87 mmol) in DMSO (1 mL) was heated at 80°C for 2 h. In another round bottom flask containing a solution of compound 12 (25.4 mg, 0.045 mmol) in dry CH$_2$Cl$_2$ (1 mL) was added a solution of carbonyl diimidazole (97.2 mg, 0.6 mmol) in dry CH$_2$Cl$_2$ (0.5 mL). The reaction was stirred at room temperature for 2 h, and transferred to the DMSO solution that was being heated. The resulting reaction was kept at the same temperature for 1.5 h. The pH was adjusted to ~5 with TFA (moist pH paper), and the CH$_2$Cl$_2$ was evaporated under reduced pressure. The residue was diluted with water and injected on to the HPLC column. Preparative C$_{18}$ column, λ = 240 nm, 6 mL/min, gradient: 0'-10': 20% B; 10'-70': 70% B. The product as the TFA salt (26.8 mg,

74.7%) eluted between 43.8% B. ^{1}H-NMR (300 MHz, d$_4$-MeOH) δ 7.9 (d, 1H); 7.5 (m, 1H); 7.35 (dd, 1H); 6.2 (m, 1H); 4.6 (bd, 2H); 4.45 (dd, 1H); 4.3 (m, 1H); 3.1-3.45 (m, 7H); 2.9 (m, 3H); 2.75 (dd, 1H); 2.2 (m, 2H); 1.25-1.8 (m, ·22H). ESI-MS (M+H$^+$)$^+$: 686.3.

Compound 22. The same procedure as described for compound 17 was used. The following amounts were used: Compound 21 (37.5 mg, 0.047 mmol); iodoacetic anhydride (25 mg, 0.07 mmol), DIPEA (16.3 mg, 0.094mmol), and dry THF (0.6 mL). The product was purified by HPLC. Preparative C$_{18}$ column, λ = 240 nm, 6 mL/min, gradient: 0'-10': 20% B; 10'-70': 70% B. The product (compound 24)(24.5 mg, 61.3%) eluted between 50.8-53% B. ESI-MS (M+H$^+$)$^+$: 848.0; (M+Na$^+$)$^+$: 876.5.

Compound 23. The same procedure as described for compound 18 was used. The following amounts were used: Compound 22 (24.5 mg, 0.029 mmol); TFA (160 µL), and CHCl3 (240 µL). The TFA salt of the product was obtained in 94.8% yield (23.6 mg). ^{1}H-NMR (300 MHz, d$_4$-MeOH) δ 8.0 (d, 1H); 7.8 (bs, 1H); 7.6 (dd, 1H); 6.1 (m, 1H); 4.5 (m, 1H); 4.4 (bd, 2H); 4.3 (dd, 1H); 4.25 (dd, 1H); 3.65 (bs, 2H); 3.55 (m, 2H); 3.15-3.35 (m, 7H); 2.95 (dd, 2H); 2.70 (m, 1H); 2.65 (dd, 1H); 2.25 (t, 2H); 1.4-1.8 (m, 13H). ESI-MS (M+H$^+$)$^+$: 754.4.

VICAT$_{SH}$(+6). The same procedure as described for VICAT$_{SH}$ was used. The following amounts were used: Compound 23 (23.6 mg, 0.027 mmol); 1% aqueous solution of formaldehyde (83 µL; 0.054 mmol); 1.2 mM methanolic NaCNBH$_3$ (23 µL; 0.054 mmol); 1.2 mM methanolic DIPEA (34

μL; 0.081 mmol); and MeOH (800 μL). The reaction was
diluted with 30% CH_3CN:water, and injected on to the HPLC
column. Preparative C_{18} column, λ = 240 nm, 6 mL/min,
gradient: 0'-10': 20% B; 10'-70': 70% B. The product
(14.8 mg, 70.8%) eluted between 36.2-39% B. ^{1}H-NMR (300
MHz, d_4-MeOH) δ 8.0 (d, 1H); 7.8 (s, 1H); 7.6 (dd, 1H);
6.15 (m, 1H); 4.45 (dd, 1H); 4.30 (dd, 1H); 3.65 (bs,
2H); 3.6 (m, 2H); 3.1-3.5 (m, 7H); 2.9-3.0 (m, 5H); 2.6
(dd, 1H); 2.2 (t, 2H); 1.2-1.8 (m, 13H). ESI-MS $(M+H^+)^+$:
768.4.

An alternate synthetic scheme for the synthesis
of intermediate 7 (Figure 1B) is described below. A
portion of the synthetic scheme is shown in Figure 6.
Note that this NBD-VICAT reagent has a 4-carbon 1,4-
diaminobutane linker between the photocleavable group and
the iodoacetyl group. It is understood that the linker
can contain an integer of carbons up to about 50. For
example, the linker can be a 4 carbon linker, NH-
$CH_2CH_2CH_2CH_2$-NH. Thus the linker can be 1, 2, 3, 4, 5, 6,
7, 8, 9, 10, 12, 14, 16, 18, 20, 30, 40 or 50 carbons, or
any intervening integer between 1 and about 50.

Compound 24. To a vial containing a solution of compound
18 (5 mg; 5.81 mmol) in MeOH (179 mL), was added 1M
acetic acid buffer pH 6.7 (76 mL), followed by 1.2 M
methanolic solution of the tert-Butyl N-(2-
oxyethyl)carbamate (19.3 mL, 23.2 mmol), and 1.2 M
methanolic solution of of $NaCNBH_4$ (19.3 mL, 23.2 mmol).
The reaction was stirred at room temperature for an hour.
The solvents were partially removed, a mixture of 20%
acetonitrile:water was added, and the reaction mixture

was injected in the HPLC. Preparative C_{18} column, $\lambda = 240$ nm, 6 mL/min, A= H_2O with 0.08% TFA, B= acetonitrile (MeCN) with 0.08% TFA, gradient: 0'-20': 20% B; 10'-80': 100% B. The TFA salt of product 24 (5.8 mg, 92.6% yield) came out between 53.8-58% B. ^{1}H-NMR (300 MHz, CHCl$_3$-d1/MeOH-d$_4$) δ 7.9 (d, 1H); 7.85 (m, 1H); 7.55 (m, 1H); 6.11 (m, 1H); 4.45 (m, 3H); 4.2 (m, 1H); 3.6 (s, 2H); 3.4-2.9 (m, 13H); 2.8 (dd, 1H); 2.6 (d, 1H); 2.2 (t, 2H); 1.65-1.25 (m, 22H). ESI-MS $(M+H^+)^+$: 891.6.0; $(M+Na^+)^+$: 913.5.

Compound 25. To a vial containing a solution of compound 24 (23.4 mg, 0.027 mmol) in CHCl$_3$ (0.4 mL) was added TFA (80 µL). The reaction mixture was stirred for 1.5 h at room temperature. Solvent was removed using a Speed-Vac. MeOH was added, and the resulting solution was concentrated in the Speed-Vac. This procedure was repeated 3 times to remove excess TFA. The TFA salt of the product (compound 25) was obtained in 98% yield (23.4 mg) as an oil. ESI-MS $(M+H^+)^+$: 763.5.

NBD-VICAT$_{SH}$. To a vial containing compound 25 (10 mg, 0.011 mmol) was added a solution of 4-Fluoro-7-nitrobenzofurazan (7.2 mg, 0.027 mmol; Dojindo Molecular Technologies, Inc., Gaithersburg MD) in DMF (640 µL), followed by diisopropylethylamine 8 µL, 0.046 mmol). After stirring for 1 hr at room temperature in the presence of 4 angstrom molecular sieves, the solvent was removed in a Speed-Vac. The residue was injected onto the HPLC column. Preparative C_{18} column, $\lambda = 500$ nm, 6 mL/min, gradient: 0'-20': 20% B; 20'-80': 100% B. An orange solid (NBD-VICAT$_{SH}$) (4.6 mg, 43.8%) eluted at 51% B.

The above example describes synthetic methods for generating VICAT reagents.

EXAMPLE II
Labeling, Detection and Cleavage of a Peptide Sample

This example describes a procedure for labeling protein samples with VICAT reagents.

Cell or tissue protein are reduced, and cysteine-containing proteins are tagged with a VICAT reagent. The sample is submitted to proteolysis, for example, with trypsin, to generate the peptide mixture. The proteolytic digestion can be carried out, for example, overnight at a suitable temperature for the protease used, such as 22-37°C for trypsin. A defined amount of one or more internal standards, peptides tagged with isotopically substituted VICAT reagent, are added to the proteolytic digest. These internal standards are readily prepared by solid-phase synthesis of the peptides of interest (matching in sequence to the peptides generated by proteolysis of the proteins of interest) and reacting the cysteine of these synthetic peptides with the isotopically heavy VICAT reagent.

The peptide mixture derived from the biological sample is subjected to isoelectric focusing (IEF) in a polyacrylamide gel strip. An adjacent IEF strip is loaded with the same internal standard that was added to the complex protein mixture. Both IEF strips are developed side-by-side, and visual inspection of the strip containing internal standard alone reveals the precise locations of the chemically identical VICAT-tagged peptide in the adjacent IEF

strip containing the complex peptide mixture. The
appropriate region of this latter strip is sliced from
the strip, and peptides in these slices are eluted with
suitable solvents, for example, 10 mM ammonia, 30%
CH_3CN. Other elution solvents can be used, as desired,
for example, aqueous buffer with detergent, chaotropic
agents, and the like, or organic solvents, which can be
used to elute peptides having low solubility in aqueous
solution. Elution yields can be monitored by the
fluorescence of VICAT-peptide conjugates. The eluted
sample mixture is neutralized, if desired, and bound to
streptavidin agarose. The VICAT-labeled and bound
peptide is released by photocleavage from the solid
phase matrix, resulting in free labeled peptide, also
called NAMETAG-peptide.

Alternatively, a relatively large amount of
one or more internal standards can be chromatographed
in a single IEF strip with the sample of interest.
Large amounts of these internal standards are provided
so that they can be visualized above a background of
VICAT-tagged, sample-derived peptides. These
visualized markers are tagged with, for example, the
+12 VICAT reagent so they can be distinguished from the
internal standard bearing, for example, a +6 VICAT tag
and the peptide coming from the example bearing, for
example, the +0 VICAT tag.

The eluted tagged-peptides are subjected to
photocleavage to cleave the VICAT reagent from the
peptide, generally so that most of the VICAT reagent is
cleaved. Photocleavage results in only a small
fragment, which bears the isotopic substitution, being
retained on the cysteine SH of the peptide. The
resulting peptides are analyzed, for example, using
tandem ESI-MS. Because the complex protein mixture
also contains a known amount of peptide standard tagged

with isotopically substituted VICAT reagent,
integration of the ion signals from the heavy and light
peptide conjugates allows accurate quantification of
the peptide of interest (see Gygi et al., *supra*, 1999).
In addition, peptide identification can be obtained by
mass spectrometric sequencing of the ESI-produced ions
by collision-induced dissociation (CID). Other mass
spectrometric methods can also be used for the
detection, quantification and identification of the
tagged peptides, based on the fact that the precise
mass and sequence of the tagged peptide are known.

Using the above described method with VICAT
reagents that contain a fluorescent or chromophoric
detectable group, all cysteine-containing peptides in
the complex cell-derived mixture are made visible by
tagging with the VICAT reagent. However, visual
localization is generally carried out only for the IEF
strip that bears the standard alone. Using chemically
identical, fluorophore-containing tags for
chromatographic marker, internal standard, and sample-
derived peptides ensures identical chromatographic
behavior. In addition to using adjacent IEF strips run
side-by-side, a single strip containing two sample
lanes can also be used. Furthermore, the VICAT-labeled
standard can be added directly into the sample as an
internal standard, particularly when differential
isotope VICAT reagents are available, such as the +0,
+6, and +12 reagents, as described above. The
differentially labeled peptides are expected to co-
migrate since it has previously been shown that ICAT-
labeled proteins precisely co-migrate in two-
dimensional gels (IEF/SDS-PAGE (sodium dodecyl sulfate
polyacrylamide gel electrophoresis))(Smolka et al.,
Mol. Cell. Proteomics 1:19-29 (2002)).

Using the above described method with VICAT
reagents that contain a radioactive detectable group,
all cysteine-containing peptides in the complex cell-
derived mixture are made visible by tagging with the
VICAT reagent in a substantially similar manner to that
described above for fluorescent or chromophoric
detectable groups. For example, standard and sample
can be run in parallel gels or parallel lanes on the
same gel. The use of a radioactive detectable group
allows the convenient addition of the standard to the
same gel strip or even directly into the sample being
analyzed, with localization of a particular sample
molecule determined by detection of the radiolabel of
the corresponding standard.

The above-described method using an internal
standard can be used to determine the absolute amount
of sample peptide in the biological sample. To
calculate the amount of sample protein from the amount
of peptide, the efficiency of proteolytic generation of
the peptide is determined. Alternatively, the amount
of sample-derived peptide can be compared to that of a
control sample, if desired.

This example describes a general protocol for
the use of a VICAT reagent to label proteins in a
sample.

EXAMPLE III
Isoelectric Focusing and ESI-MS Analysis of Peptides

This example describes the analysis of
peptides labeled with a VICAT reagent using isolectric
focusing and ESI-MS.

The peptide mixture consisting of the tagged
peptides from the protein sample mixture and the

differentially isotopically tagged internal standard
are often very complex and need to be separated prior
to ESI-MS analysis. Any of a number of peptide
separation methods can be applied to the sample, for
example, chromatography, electrophoresis, and the like.
Among the multitude of mature peptide separation
methods available, isoelectric focusing (IEF) in
polyacrylamide gels with immobilized pH gradients is
particularly useful for the following reasons: i) The
method is robust, relatively inexpensive, highly
reproducible and easily multiplexed (Righetti et al.,
Methods. Enzymol. 270:235-55 (1996)). IEF strips
covering different pH ranges are commercially available
(for example, Pharmacia-Amersham Biotech; Piscataway
NJ); ii) The method has immense separation power
(Righetti et al., *supra*, 1996), and the pH gradient,
and therefore the range of maximal separation, can be
tuned, as desired for particular experimental
applications; iii) The pI of peptides can be reasonably
well calculated from their amino acid sequences
(Bjellqvist et al., Electrophoresis 14:1023-1031
(1993)); thus, the migration position of a specific
target peptide-VICAT conjugate in the gel can be
predicted, allowing rational choice of the pH range of
the IEF used values. The tetramethylrhodamine group of
the VICAT reagent will be zwitterionic when the pH
equals the pI for most peptides, and thus the peptide
and VICAT-peptide conjugate will have similar pI
values. Furthermore, the pI value of the VICAT-peptide
conjugate can be calculated using the pKa value of the
COOH group of the tetramethylrhodamine; and vi) Peptide
recovery is achieved in high yield simply by soaking
the gel segment in an appropriate solvent such as an
aqueous buffer.

For analysis of peptide samples by IEF, a
peptide sample generated by tryptic digestion, or other

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suitable protease digestion, of the VICAT-labeled
protein sample essentially as described in Example II
(see also Gygi et al., *supra*, 1999). Internal standard
peptide(s) are added to the VICAT-labeled digest with
soluble carrier ampholytes to a final concentration of
0.5% and applied to a rehydration cassette (Pharmacia-
Amersham Biotech). The gel is allowed to swell for 4h.
The gel strips are transferred to the electrophoresis
chamber and focused at a final voltage of 8000V for a
total of 115,000 voltßhours. The voltage is slowly
ramped up to avoid peptide precipitation and local
overheating of the gel. A second identical gel strip
is loaded with the external tagged peptide standard(s)
alone for the localization of the target peptide(s) in
the gel. The gel strips are subjected either to visual
inspection or to fluorescence imaging, if needed for
increased sensitivity. The gel segments containing the
target peptide(s) are extracted and the isolated
peptides are bound to the affinity matrix. The
affinity isolated peptides are subjected to
photocleavage to remove the fluorescent tag and release
the NAMETAG-peptides having the isotopic label. The
peptide-NAMETAG conjugates are analyzed by combined
microbore-HPLC/ESI-MS using the standard protocols and
software tools developed for quantitative protein
profiling by the ICAT method (Han et al., Nat.
Biotechnol. 19:946-951 (2001); Gygi et al., *supra*,
1999).

If the affinity reagent is avidin, the
extracted peptides are bound to streptavidin modified
agarose beads or streptavidin modified magnetic beads
using standard protocols and washed. NAMETAG peptides
are recovered from the solid support by photo cleavage
of the linker. The beads re re-suspended in of 0.2 M
Tris, pH 8.0, 10 mM EDTA with 2% β-mercaptoethanol to
prevent methionine oxidation during photo-cleavage.

Light from a Blak-Ray longwave UV lamp (VWR) is
filtered by 10% of copper (II) sulfate solution (1 cm
path length) and used to illuminate the beads for 2
hours. Beads are occasionally agitated to ensure
uniform light illumination. The recovered peptides are
analyzed by LC-MS/MS using the standard protocols and
software tools developed for quantitative protein
profiling (Gygi et al., *supra*, 1999; Han et al., Nat.
Biotechnol. 19:946-951 (2001)).

       This example describes protocols for IEF and
ESI-MS analysis of VICAT-labeled peptides.

                          EXAMPLE IV
           Dystrophin Analysis Using VICAT Reagents

       This example describes the use of the VICAT
technology for the quantification of dystrophin and its
structural variants, which is clinically relevant for
the diagnoses of Duchenne's and Becker's muscular
dystrophy.

       The distribution of pI values for the full
set of cysteine-containing, tryptic peptides derived
from the human proteome is calculated. With current
genome annotation, ~33,000 proteins are predicted,
giving ~230,000 cysteine-containing, tryptic peptides.
It is expected that far fewer proteins will be
expressed in any given cell type. The distribution of
pI values is not uniform; clusters of up to a few
thousand peptides exist. Thus, it is useful to search
for those dystrophin-derived peptides that will migrate
in the IEF strip in a noncrowded region. There are 8
dystrophin-derived, cysteine-containing, tryptic
peptides that will migrate in relatively non-crowded
regions containing <50 peptides. Also, the pH gradient

in the crowded regions can be flattened, providing better separation.

The VICAT method allows selective detection of segments of dystrophin, which is useful for the diagnosis of variants of muscular dystrophy caused by dystrophin deletions. The amount of dystrophin present in total skeletal muscle protein can also be determined. Initial studies are carried out with the crude surface membrane fraction prepared from skeletal muscle using a simple 3-step differential centrifugation procedure (Ohlendieck et al., J. Cell Biol. 112:135-148 (1991)). Approximately 1% of the total protein in this fraction is dystrophin, whereas this protein constitutes 0.002% of total skeletal muscle protein (Ohlendieck and Campbell, FEBS Lett. 283:230-234 (1991)). A 100 mg sample of skeletal muscle obtained by needle biopsy contains ~0.5 μg of dystrophin (~1 pmol), which is more than sufficient for analysis. For example, 10-50 fmol of peptide can be routinely detected when this amount is applied to microbore-HPLC/ESI-MS system.

For analysis of dystrophin in muscle sample, proteins in the membrane fraction are solubilized by boiling in 50 mM Tris-HCl, pH 8.3, 5 mM EDTA, and either 0.5% SDS or 0.05% SDS/6 M urea. These buffers have been shown to work well for ICAT analysis of microsomal proteins (Han et al., Nat. Biotechnol. 19:946-951 (2001); Smolka et al., Anal. Biochem. 297:25-31 (2001)). Protein disulfides are reduced by treatment with 5 mM tributyl phosphine for 30 min at 37°C (Smolka et al., supra, 2001), and cysteines are tagged with 200 nM to 1 mM VICAT reagent, depending on the amount of peptides in the sample to be analyzed, for 90 min at room temperature. The sample is diluted 10-fold to reduce the concentration of denaturants, a

known amount of internal standard (for example,
synthetic dystrophin peptide tagged with VICAT(+6)) is
added, and the sample is treated with trypsin overnight
at 37°C. VICAT-peptide conjugates can be isolated
using solid-phase, monomeric avidin as described
previously for ICAT reagents (Gygi et al., *supra*,
1999). IEF and microbore-HPLC/ESI-MS are carried out
as described above. If peptide sample and IEF marker
are run in separate lanes of the same IEF strip, the
VICAT(+12) reagent can optionally be used to prepare
the IEF marker in case some of it diffuses into the
adjacent IEF lane containing the peptide mixture.
Alternatively, the VICAT-labeled peptide standards are
added to the sample molecules for resolution in a
single IEF lane.

        Alternatively, following reaction of sample
peptides with a VICAT reagent, salt and detergent are
removed by ion exchange chromatography, such as cation
exchange chromatography, and step elution. This step
can also serve to exchange the buffer composition for
further manipulations. VICAT-labeled peptides in the
sample mixture are resolved by IEF. The location of
VICAT-labeled sample peptides are visualized using
VICAT labeled standard peptides resolved in parallel or
as an internal standard added to the sample mixture.
Resolved VICAT-labeled peptides of interest are
absorbed onto streptavidin beads. Photocleavage is
carried out on the affinity isolated peptides,
resulting in release of NAMETAG-peptide conjugate(s),
which contain the isotope tag. The released NAMETAG-
peptide conjugate(s) are analyzed by LC-MS/MS.
Digestion with a protease such as trypsin can
optionally be included at various steps, such as before
or after reacting with the VICAT reagent, before or
after resolving sample molecules, or before or after
binding to the affinity resin.

The quantification of dystrophin using VICAT
reagents can be carried out using mouse skeletal
muscle. Frozen tissue is commercially available (Pel-
Freeze Inc.; Rogers AR). Of the 20 cysteine-containing,
tryptic peptides of >5 amino acids derived from
dystrophin, 16 have identical sequences for mouse and
human proteins, and 4 have nearly identical sequences.
Skeletal muscle from the mdx mouse, which lacks
dystrophin, can also be used (Sicinski et al., Science
244:1578-1580 (1989)). After validation of the VICAT
assay using mouse tissue, human muscle samples are
obtained using Institutional Review Board approved
protocols and analyzed as described above.

This example describes the analysis of
dystrophin using a VICAT-reagent.

EXAMPLE V

Protein Detection Using VICAT$_{SH}$ Reagents

This example describes protein detection in a
crude extract using VICAT reagents.

Experiments were performed to quantify a
specific protein in a complex protein mixture using VICAT
reagents. Briefly, a crude cell lysate derived from
insect cells (Spodoptera frugiperda, Sf9) was spiked with
different amounts of recombinant human group V secreted
phospholipase A_2 (hGV). Sf9 cells provide a good negative
control since they lack hGV. The cysteine-containing,
tryptic peptide derived from hGV, SYNPQYQYFPNILCS (SEQ ID
NO:1), was synthesized and derivatized with VICAT$_{SH}$(+6) to
make the internal standard and with ^{14}C-VICAT$_{SH}$(-28) to

make the chromatographic standard. Sf9 cells were lysed
in denaturing buffer containing urea and CHAPS, and the
lysate was spiked with various amounts of recombinant hGV
and with known amounts of internal standard and
chromatographic marker. The sample containing 200 μg of
cell protein was submitted to IEF on a commercial gel
strip (Amersham; Piscataway NJ). A 0.5-cm section of the
strip was cut into 5 pieces, and peptides in each slice
were eluted with $CH_3CN/H_2O/CF_3COOH$. From 5 such IEF
strips, it was found that the yield of recovered
radioactivity (due to the chromatographic marker), based
on recovered dpm versus dpm applied to the IEF strip,
ranged from 50-60%. All of the recovered radioactivity
was found either in a single 1 mm IEF slice or in 2
adjacent slices, demonstrating that tagged peptides focus
in a narrow zone. VICAT-tagged peptides were captured
with streptavidin-Agarose and released by photocleavage
in 60-70% yield (based on test experiments using mass
spectrometry with an internal standard for
quantification).

The material from the photocleavage reaction
was submitted to combined capillary-LC/electrospray
ioniziation mass spectrometry using an LCQ instrument
(Finnigan; Austin TX). The ion trap was operated in
"selected reaction mode" (SRM, Finnigan). The ion trap
collects a range of ions and ejects all ions except those
of desired m/z values. In this case, only precursor ions
of m/z = 981-989 AMU are held in the trap. This range
includes the precursor ion from the hGV-derived, tagged
peptide (m/z = 984 for the +2 ion) and the precursor ion
for the internal standard (m/z = 987 for the +2 ion).
Trapped ions were subjected to CID. In SRM, a set of

fragment ions with desired m/z are held in the trap, all other ions are ejected. SRM was used to monitor 5 y-ion fragments derived from the precursor ion from the tagged hGV-derived peptide, which contains the light isotope tag. SRM was also used to monitor 5 y-ion fragments derived from the precursor ion from the internal standard, which contains the heavy isotope tag.

Figure 7, left panel, shows the abundance of the doubly charged y_{12} ion (the most intense y-ion, m/z = 800.8) for the light peptide (derived from the VICAT$_{SH}$-tagged hGV protein) as a function of capillary-LC retention time. Figure 7, right panel, shows the doubly charged y_{12} ion for the heavy peptide (m/z = 803.8, derived from the internal standard). As expected, the two ions are found to co-elute during capillary-LC. For this sample, Sf9 cell lysate (200 µg protein) was spiked with 500 fmol of hGV protein and 500 fmol internal standard. Thus, the expected ratio of hGV-derived and internal standard-derived tagged peptides is 1:1; the observed ratio was 1.1:1. No peak above the noise was observed at m/z = 800.8 when hGV was omitted from the Sf9 cell lysate. Judging from the excellent signal-to-noise seen in Figure 7, 20-fold less material could be observed. Thus, a conservative estimate of the detection limit is 25 fmol of hGV per 200 µg of cell lysate protein. Antiserum to hGV can detect down to ~0.5 ng (33 fmol) of hGV in a western blot using ECL detection with a maximum loading capacity on one lane of an SDS-PAGE mini-gel of 20 µg cell lysate protein (Degousee et al. _J. Biol. Chem._ 277:5061-5073 (2002)).

These results indicate that the VICAT-based
method for detecting hGV is ~14-fold more sensitive than
the western blot (0.12 fmol per microgram of lysate
protein versus 1.7 fmol per microgram). Even higher
sensitivity can be achieved with the VICAT method.
Firstly, up to 2.5-fold higher amounts of protein (500
µg) can be loaded onto the IEF strip. Secondly, higher
ion detection sensitivity can be achieved using a mass
spectrometer with a quadrupole precursor ion selector.

EXAMPLE VI

High Throughput Proteome Analysis Using of VICAT Reagents

This example describes high throughput analysis
of protein samples using VICAT reagents.

VICAT reagents can be used to analyze protein
samples using capillary-LC coupled directly to the
electrospray interface of an ion trap mass spectrometer
(Finnigan LCQ). However, owing to the relatively long
combined capillary-LC/mass spectrometry runs (~2 hr per
sample), this method is not very high-throughput. To
increase the sample throughput, the use of MALDI-MS/MS
for the analysis of VICAT peptides has been explored.
The samples are separated as for the LC-ESI-MS method.
However, instead of spraying the peptides into the mass
spectrometer in real time, they are deposited on the
MALDI sample plate together with matrix, using an
automatic MALDI plate spotter. The plates are analyzed
in a MALDI-MS/MS system. The increase in sample
throughput is realized by the following features of the
approach: Firstly, peptide separation and analysis are
uncoupled. Therefore, several separations can be carried

out in parallel and analyzed by a single, fast MALDI-
MS/MS system.   Secondly, only the segments of the
capillary-LC solvent gradient in which the VICAT peptides
are expected to elute need to be interrogated by the mass
spectrometer.   Thirdly, data analysis (that is, the
number of laser shots per sample) can be optimized for
each VICAT peptide pair, thus minimizing data acquisition
time per spot.   Furthermore, the samples prepared in this
way represent a quasi permanent sample archive, allowing
re-analysis of samples, if required.

An automated, multiplexed capillary-LC system
has been developed and interfaced with a ProBot MALDI
plate spotter.   The system operates four capillary
columns from a single binary solvent delivery system and
therefore supports the concurrent separation and
deposition on the sample plate of four samples at a high
degree of reproducibility (Lee et al., Anal. Chem.
74:4353-4360 (2002)).   MALDI analysis of the few spots
collected per sample is extremely fast.   Assuming that
100 laser shots are required per sample spot to acquire
high quality MS spectra and that VICAT peptide pairs are
measured in 5 consecutive plate spots, only 2.5 sec of
data acquisition time is needed using a 200 Hz laser.

Two different MALDI-MS/MS systems are available
for sample analysis.   The first is equipped with an
analytical quadrupole, a quadrupole collision cell and an
orthogonal time-of-flight detector (MALDI QqTOF, Model
QSTAR by ABI/SCIEX; Applied Biosystems, Foster City CA)
and the second is a TOF-TOF system (Model 4700, ABI;
Applied Biosystems).   Both instruments contain a true
collision cell for the generation of MS/MS spectra in

cases where VICAT peptides need to be sequenced. Using
the QqTOF instrument, instrument control protocols have
been developed that allow detection and quantification of
isotopically labeled peptide pairs and subsequent
sequencing of those peptides that show a specific
quantitative ratio (abundance dependent precursor ion
selection). (Griffin et al., Anal. Chem. 75:867-874
(2003). The capacity of this instrument to automatically
acquire MS spectra from each or selected spots on the
sample plate, to detect peptide pairs and to quantify
their abundance is ideally suited for the analysis of the
VICAT samples. Similar instrument control protocols are
currently being developed for the faster MALDI TOF-TOF
instrument (200 Hz laser compared to 29 Hz laser for the
QqTOF instrument).

The analytical quadrupole can be set in a mass-
selective mode to allow passage of only the m/z and (m +
6)/z ions, corresponding to the sample-derived and
internal standard peptides. In the case of the MALDI
TOF-TOF instrument, precursor ion selection is achieved
by a timed gate in the first TOF analyzer. The
resolution of the TOF analyzer for gating of precursor
ions is not as high as that for the quadrupole of the
QqTOF. For the MALDI TOF-TOF, it is about 5-10 AMU and
somewhat mass dependent; however, quantification is based
on fragment ions, which allows further exclusion of
peptides that do not bear the isotope tag.

The high throughput analysis described above
makes it possible for many biochemical laboratories to
use the VICAT method to quantify specific proteins in
complex protein mixtures in a relatively high throughput

manner. The high throughput applications of VICAT
technology such as the analysis of differential protein
splicing, the analysis of post-translational
modifications, and the quantification of specific
proteins without the need for highly specific antibodies
may become a routinely used laboratory tool.

EXAMPLE VII

Modification of Peptides to Incorporate Sulfhydryl Groups

This example describes modification of peptides
to incorporate sulfhydryl groups, which can be used to
convert amino groups of peptides that do not contain
cysteine to SH groups for subsequent reaction with SH-
selective VICAT reagents.

The amino groups of peptides are converted to
sulfhydryl groups that can be reacted with sulfhydryl
reacting compounds such as VICAT$_{SH}$ reagents. The methods
can be applied to single peptides as well as complex
mixtures of proteins. For example, almost all tryptic
peptides contain an N-terminal amino group and a subset
contain a C-terminal lysine that has an ε-amino group.
The peptides are reacted with the reagent SATA, which is
the N-hydroxysuccinimidyl ester of S-acetyl thioacetic
acid (AcetylS-CH$_2$-CONHS). Reaction of a peptide with SATA
gives the peptide in which AcetylS-CH$_2$CO is attached to
the amino groups via an amide linkage: AcetylS-CH$_2$-CONH-
peptide. The modified peptide is then treated with
hydroxylamine (NH$_2$OH) to cleave the thiol ester, resulting
in each amino group containing a thioacetyl group: HS-
CH$_2$-CONH-peptide. The peptide can now be captured with
sulfhydryl reacting reagents such as VICAT$_{SH}$ reagents,

that is, the iodoacetyl group of VICAT$_{SH}$ reagents can
react with this newly introduced thiol. A VICAT$_{SH}$ reagent
can thus be used to label cysteine-containing peptides as
well as peptides containing amino groups that are
converted to sulfhydryl groups.

Briefly in more detail, immediately before
reaction, AcetylS-CH$_2$-CO-NHS (SATA) is dissolved in dry
DMF to 40 mM final concentration. To an eppendorf tube
containing 14 mL of Buffer (50:50 DMF: 0.1 M HEPES; pH
8.14) was added 1 mL of the target peptide (1 mM solution
in purified water such as MilliQ water) followed by 5 mL
of SATA (40mM solution in DMF). The reaction was kept at
room temperature for 2h, and 10 mL of the deacetylation
solution (50 mM sodium phosphate, 25 mM EDTA, 0.5 M
Hydroxylamine·HCl adjusted to pH 7.5) was added.
Deacetylation was allowed to proceed for 2 hr at room
temp.

The peptide mixture can be freed of excess
reagents by diluting the above reaction mixture with
water containing 0.1% TFA and loading the mixture onto a
C$_{18}$ reverse phase cartridge. The cartridge is washed
with several volumes of water/0.1% TFA, and the peptides
are eluted from the cartridge with acetonitrile/0.1% TFA.
After removal of solvent *in vacuo*, the peptide mixture
can be submitted to modification with VICAT$_{SH}$ reagents as
described above.

The above-described procedure leads to quantitative
conversion of peptide amino groups (N-terminal amino
group or epsiolon amino group of lysine) to NHCOCH$_2$SH
groups. No modification of other amino acids has been

observed when this procedure is applied to more than a
dozen different peptides that have been tested.

        Throughout this application various
publications have been referenced. The disclosures of
these publications in their entireties are hereby
incorporated by reference in this application in order
to more fully describe the state of the art to which
this invention pertains. Although the invention has
been described with reference to the examples provided
above, it should be understood that various
modifications can be made without departing from the
spirit of the invention.

What is claimed is:

1. A reagent comprising an affinity tag, a detectable moiety, a linker, an isotope tag and a reactive group.

2. The reagent of claim 1, wherein the detectable moiety is a fluorophore or chromophore.

3. The reagent of claim 1, wherein the detectable moiety is radioactive.

4. The reagent of claim 3, wherein the detectable moiety comprises a radioactive atom selected from the group consisting of ^3H, ^{14}C, ^{32}P, ^{35}S and ^{125}I.

5. The reagent of claim 1, wherein said linker is cleavable.

6. The reagent of claim 5, wherein said linker comprises a photocleavable moiety, a chemically cleavable moiety, or an enzymatically cleavable moiety.

7. The reagent of claim 1, wherein said affinity tag is selected from the group consisting of biotin or a derivative thereof, glutathione, maltose, poly(His), and an epitope tag.

8. The reagent of claim 1, wherein said reactive group is selected from the group consisting of a sulfhydryl reactive group, an amine reactive group, and a carboxyl reactive group.

9.   The reagent of claim 1, said reagent having the general formula:

$$A \\\diagdown \\ \diagup \\ D \diagup \quad L\text{-}T\text{-}R$$
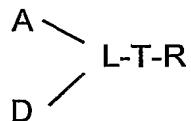
wherein A is an affinity tag, D is a detectable moiety, L is a linker, T is an isotope tag, and R is a reactive group.

10.   The reagent of claim 9, wherein the detectable moiety is a fluorophore or chromophore.

11.   The reagent of claim 9, wherein the detectable moiety is radioactive.

12.   The reagent of claim 11, wherein the detectable moiety comprises a radioactive atom selected from the group consisting of ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{125}I.

13.   The reagent of claim 9, wherein said linker is cleavable.

14.   The reagent of claim 13, wherein said linker comprises a photocleavable moiety, a chemically cleavable moiety, or an enzymatically cleavable moiety.

15.   The reagent of claim 9, wherein said affinity tag is selected from the group consisting of biotin or a derivative thereof, glutathione, maltose, poly(His), and an epitope tag.

16.   The reagent of claim 9, wherein said reactive group is selected from the group consisting of a sulfhydryl reactive group, an amine reactive group, and a carboxyl reactive group.

17.   A kit comprising the reagent of claim 1.

18.   The kit of claim 17, wherein the detectable moiety of said reagent is a fluorophore or chromophore.

19.   The kit of claim 17, wherein the detectable moiety of said reagent is radioactive.

20.   The kit of claim 19, wherein the detectable moiety of said reagent comprises a radioactive atom selected from the group consisting of ^3H, ^{14}C, ^{32}P, ^{35}S and ^{125}I.

21.   The kit of claim 17, wherein said linker of said reagent is cleavable.

22.   The kit of claim 21, wherein said linker of said reagent comprises a photocleavable moiety, a chemically cleavable moiety, or an enzymatically cleavable moiety.

23.   The kit of claim 17, wherein said affinity tag of said reagent is selected from the group consisting of biotin or a derivative thereof, glutathione, maltose, poly(His), and an epitope tag.

24.   The kit of claim 17, wherein said reactive group is selected from the group consisting of a sulfhydryl reactive group, an amine reactive group, and a carboxyl reactive group.

25.   A method of labeling a polypeptide in a sample, comprising contacting a sample with the reagent of claim 1 under conditions allowing the reactive group to bind to one or more polypeptides in said sample.

26.   The method of claim 25, wherein the detectable moiety of said reagent is a fluorophore or chromophore.

27.   The method of claim 25, wherein the detectable moiety of said reagent is radioactive.

28.   The method of claim 27, wherein the detectable moiety of said reagent comprises a radioactive atom selected from the group consisting of ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{125}I.

29.   The method of claim 25, wherein said linker of said reagent is cleavable.

30.   The method of claim 29, wherein said linker of said reagent comprises a photocleavable moiety, a chemically cleavable moiety, or an enzymatically cleavable moiety.

31.   The method of claim 25, wherein said affinity tag is selected from the group consisting of biotin or a derivative thereof, glutathione, maltose, poly(His), and an epitope tag.

32.   The method of claim 25, wherein said reactive group is selected from the group consisting of a sulfhydryl reactive group, an amine reactive group, and a carboxyl reactive group.

33.   A method of isolating a polypeptide in a sample, comprising the steps of:

(a)   contacting a sample with the reagent of claim 1 under conditions allowing the reactive group to bind to and react with one or more polypeptides in said

sample, thereby tagging one or more polypeptides with said reagent;

(b)   resolving the polypeptides in said sample;

(c)   visualizing the polypeptides tagged with said reagent;

(d)   contacting said tagged polypeptides with a capture moiety for said affinity tag; and

(e)   isolating said tagged polypeptides.

34.   The method of claim 33, wherein said linker of said reagent is cleavable.

35.   The method of claim 34, further comprising the step of cleaving said linker.

36.   The method of claim 35, wherein said linker of said reagent comprises a photocleavable moiety, a chemically cleavable moiety, or an enzymatically cleavable moiety.

37.   The method of claim 33, wherein the detectable moiety of said reagent is a fluorophore or chromophore.

38.   The method of claim 33, wherein the detectable moiety of said reagent is radioactive.

39.   The method of claim 38, wherein the detectable moiety comprises a radioactive atom selected from the group consisting of ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{125}I.

40. The method of claim 33, wherein said affinity tag of said reagent is selected from the group consisting of biotin or a derivative thereof, glutathione, maltose, poly(His), and an epitope tag.

41. The method of claim 33, wherein said reactive group of said reagent is selected from the group consisting of a sulfhydryl reactive group, an amine reactive group, and a carboxyl reactive group.

42. A method of identifying a polypeptide in a sample, comprising the steps of:

(a) contacting a sample with the reagent of claim 1 under conditions allowing the reactive group to bind to and react with one or more polypeptides in said sample, thereby tagging one or more polypeptides with said reagent;

(b) resolving the polypeptides in said sample;

(c) visualizing the one or more polypeptides tagged with said reagent;

(d) contacting said one or more tagged polypeptides with a capture moiety for said affinity tag;

(e) isolating said one or more tagged polypeptides;

(f) cleaving the linker of said reagent to release said one or more tagged polypeptides; and

(g) identifying a released tagged polypeptide.

43.   The method of claim 42, wherein said identifying step is performed using mass spectrometry.

44.   The method of claim 43, further comprising quantifying the amount of said identified polypeptide.

45.   The method of claim 42, wherein said linker of said reagent is cleavable.

46.   The method of claim 45, further comprising the step of cleaving said linker.

47.   The method of claim 46, wherein said linker of said reagent comprises a photocleavable moiety, a chemically cleavable moiety, or an enzymatically cleavable moiety.

48.   The method of claim 46, wherein the detectable moiety is a fluorophore or chromophore.

49.   The method of claim 42, wherein the detectable moiety is radioactive.

50.   The method of claim 49, wherein the detectable moiety comprises a radioactive atom selected from the group consisting of ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{125}I.

51.   The method of claim 42, wherein said affinity tag of said reagent is selected from the group consisting of biotin or a derivative thereof, glutathione, maltose, poly(His), and an epitope tag.

52.   The method of claim 42, wherein said reactive group is selected from the group consisting of

a sulfhydryl reactive group, an amine reactive group, and a carboxyl reactive group.

53.   A method of identifying a polypeptide in a sample, comprising the steps of:

(a)   contacting a sample with the reagent of claim 1 under conditions allowing the reactive group to bind to and react with one or more polypeptides in said sample, thereby labeling one or more polypeptides with said reagent;

(b)   cleaving the polypeptides in said sample to generate peptide fragments;

(c)   adding one or more peptide standards to said sample, wherein said peptide standards correspond to peptides generated from cleaving sample polypeptides as in step (b) and are labeled with an isotopically distinct version of said isotope tag;

(d)   resolving the labeled peptides in said sample;

(e)   visualizing the labeled peptides;

(f)   contacting said labeled peptides with a capture moiety for said affinity tag;

(g)   isolating said labeled peptides;

(h)   cleaving the linker of said reagent to release said isolated peptides; and

(i)   identifying a released peptide.

54.   The method of claim 53, wherein said identifying step is performed using mass spectrometry.

55.   The method of claim 53, further comprising quantifying the amount of said identified peptide.

56.   The method of claim 53, wherein said linker of said reagent is cleavable.

57.   The method of claim 56, further comprising the step of cleaving said linker.

58.   The method of claim 57, wherein said linker of said reagent comprises a photocleavable moiety, a chemically cleavable moiety, or an enzymatically cleavable moiety.

59.   The method of claim 53, wherein the detectable moiety of said reagent is a fluorophore or chromophore.

60.   The method of claim 53, wherein the detectable moiety of said reagent is radioactive.

61.   The method of claim 60, wherein the detectable moiety comprises a radioactive atom selected from the group consisting of ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{125}I.

62.   The method of claim 53, wherein said affinity tag of said reagent is selected from the group consisting of biotin or a derivative thereof, glutathione, maltose, poly(His), and an epitope tag.

63.   The method of claim 53, wherein said reactive group of said reagent is selected from the

group consisting of a sulfhydryl reactive group, an
amine reactive group, and a carboxyl reactive group.

64.   The method of claim 53, wherein the
polypeptides in said sample are cleaved with trypsin.

65.   A method of diagnosing a disease,
comprising the steps of:

(a)   contacting a sample from a patient with
the reagent of claim 1 under conditions allowing the
reactive group to bind to and react with one or more
polypeptides in said sample, thereby labeling one or
more polypeptides with said reagent;

(b)   cleaving the polypeptides in said sample
to generate peptide fragments;

(c)   adding one or more peptide standards to
said sample, wherein said peptide standards correspond
to peptides generated from cleaving sample polypeptides
as in step (b), are labeled with an isotopically
distinct version of said isotope tag, and are derived
from a polypeptide exhibiting aberrant expression in
said disease;

(d)   resolving the labeled peptides in said
sample;

(e)   visualizing the labeled peptides;

(f)   contacting the labeled peptides with a
capture moiety for said affinity tag;

(g)   isolating said labeled peptides;

(h)   cleaving the linker of said reagent to release said isolated peptides;

(j)   quantifying said released peptides; and

(k)   comparing the amount of sample peptide to standard peptide in said released peptides, wherein the amount of sample peptide is correlated with the diagnosis of said disease.

66.   The method of claim 65, wherein said disease is muscular dystrophy.

67.   The method of claim 66, wherein said disease is Duchenne muscular dystrophy or Becker muscular dystrophy.

68.   The method of claim 65, wherein said disease is prostate cancer.

69.   The method of claim 65, wherein said identifying step is performed using mass spectrometry.

70.   The method of claim 65, wherein said linker is cleavable.

71.   The method of claim 70, further comprising the step of cleaving said linker.

72.   The method of claim 65, wherein said linker of said reagent comprises a photocleavable moiety, a chemically cleavable moiety, or an enzymatically cleavable moiety.

73.   The method of claim 65, wherein the detectable moiety of said reagent is a fluorophore or chromophore.

74. The method of claim 65, wherein the
detectable moiety of said reagent is radioactive.

75. The method of claim 74, wherein the
detectable moiety comprises a radioactive atom selected
from the group consisting of ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{125}I.

76. The method of claim 65, wherein said
affinity tag of said reagent is selected from the group
consisting of biotin or a derivative thereof,
glutathione, maltose, poly(His), and an epitope tag.

77. The method of claim 65, wherein said
reactive group of said reagent is selected from the
group consisting of a sulfhydryl reactive group, an
amine reactive group, and a carboxyl reactive group.

78. The method of claim 65, wherein the
polypeptides in said sample are cleaved with trypsin.
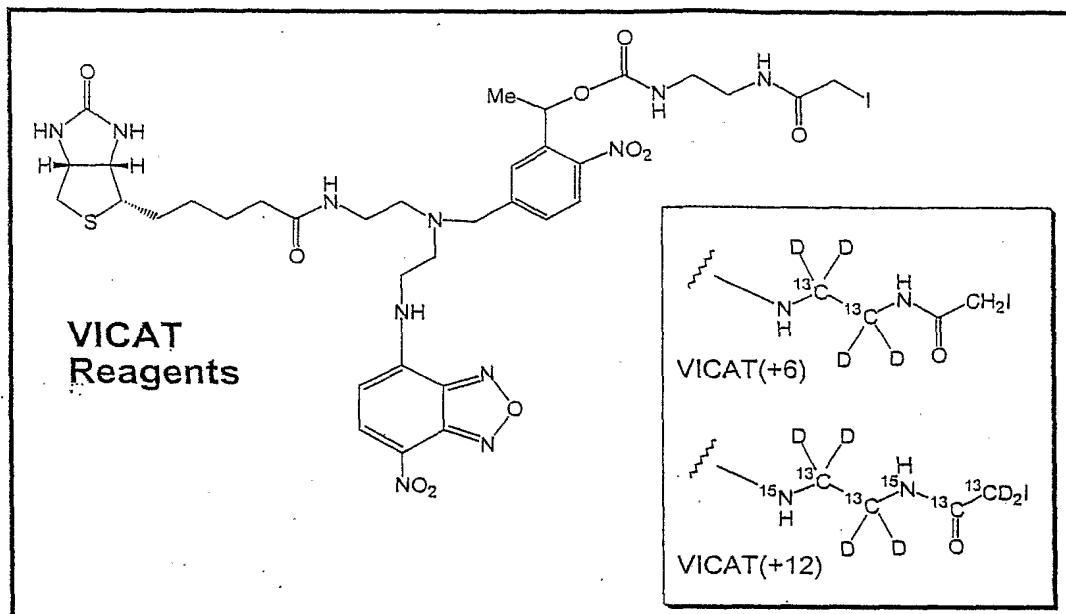
FIGURE 1A

Reaction Scheme

FIGURE 1B

3/10

20% TFA/CHCl₃
quant.

6

DMF, DIPEA
43.8%

7

TFA⁻ + NH₃

8

FIGURE 1B CONT.

FIGURE 2

FIGURE 3

^{14}C-Acetyl-VICAT
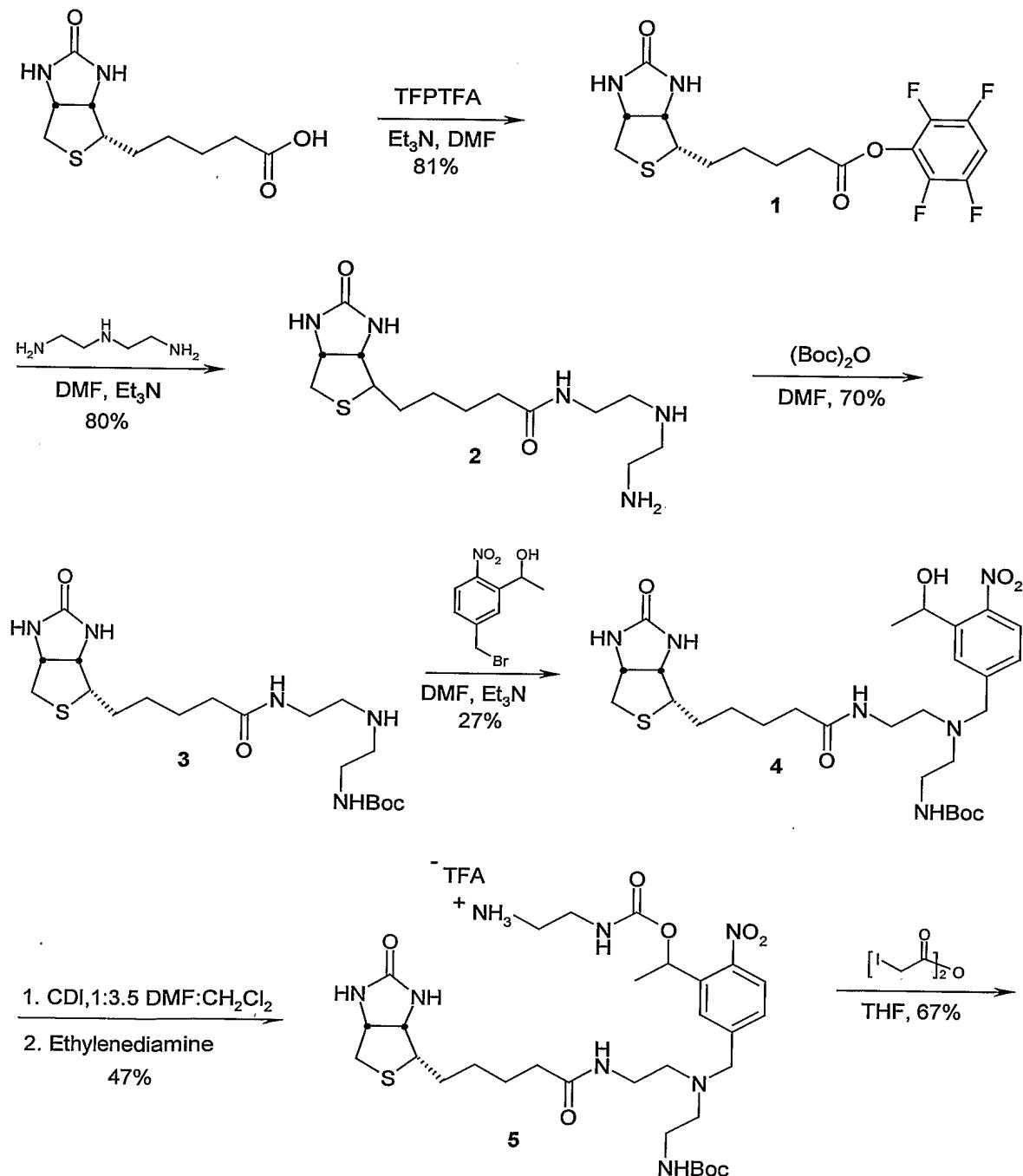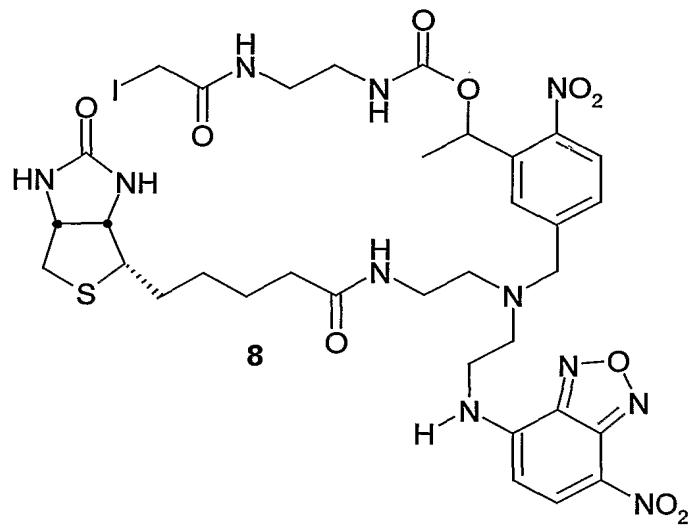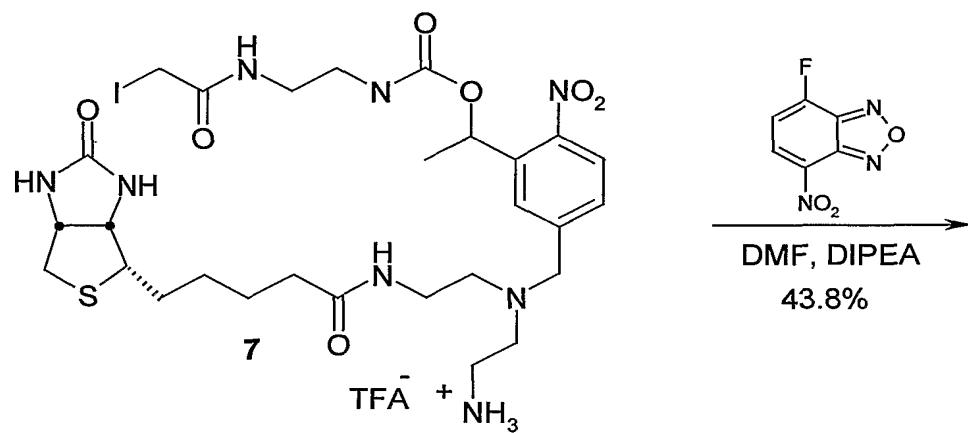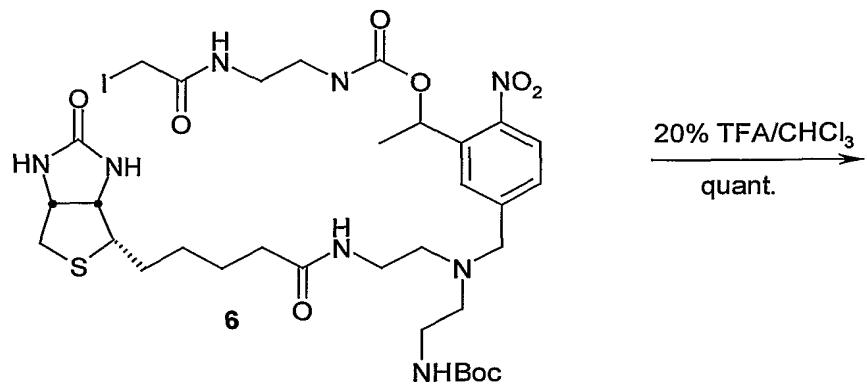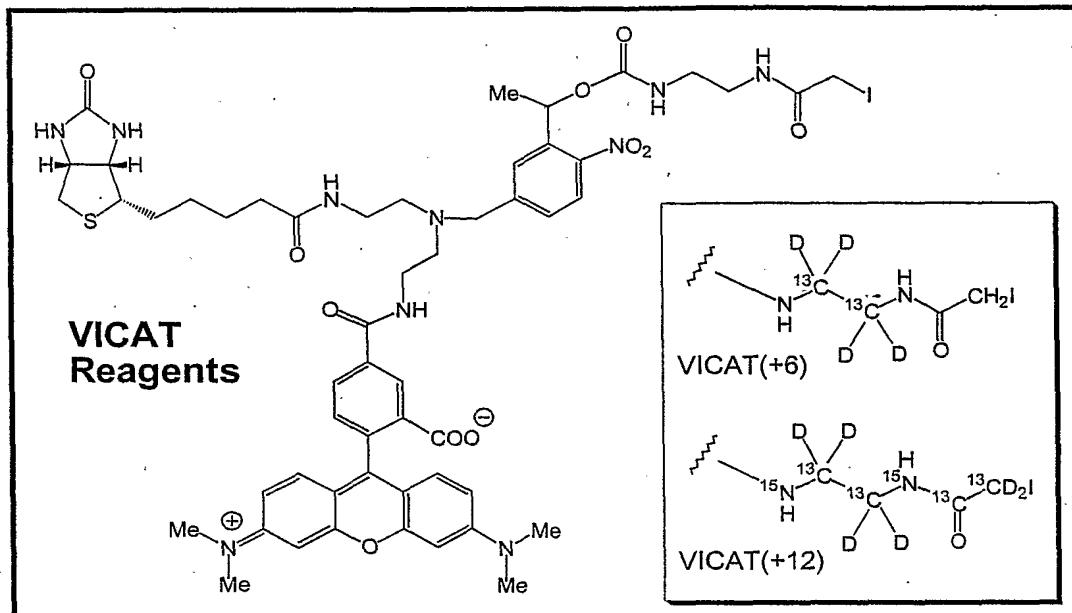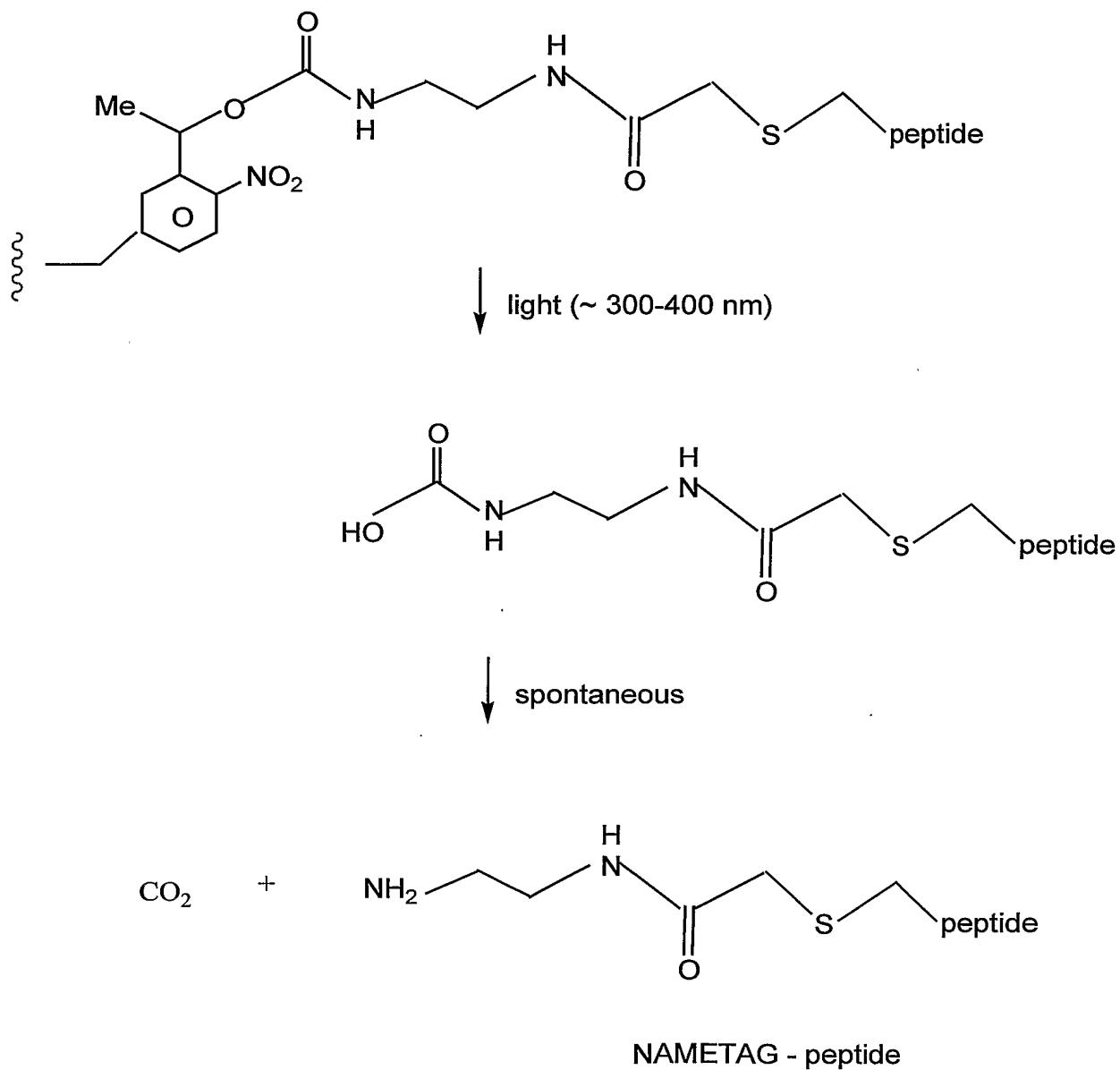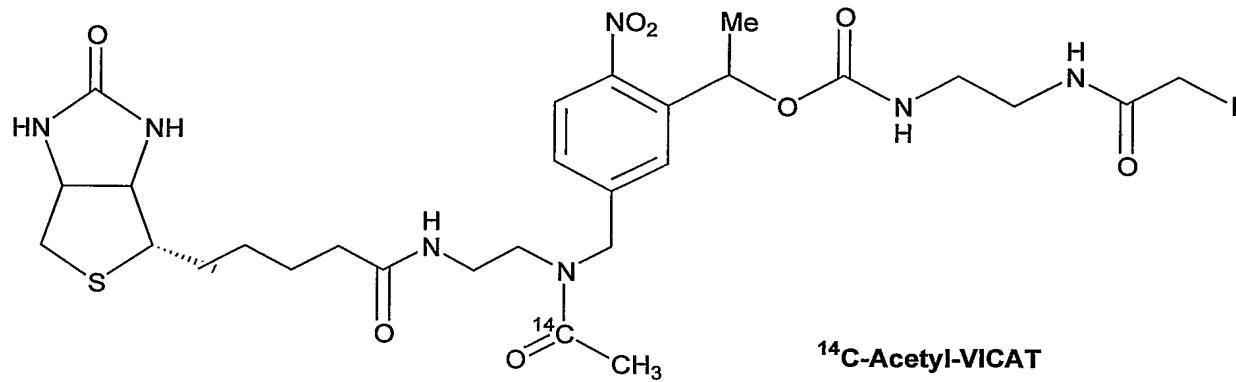
FIGURE 4A

7/10

FIGURE 4B

8/10

Figure 5

1. BOC-NH-CH₂-CHO
   NaCNBH₄
   MeOH, AcOH, H₂O
2. TFA, CHCl₃

18

NBD-F
DMF, (i-Pr) 2NEt

25
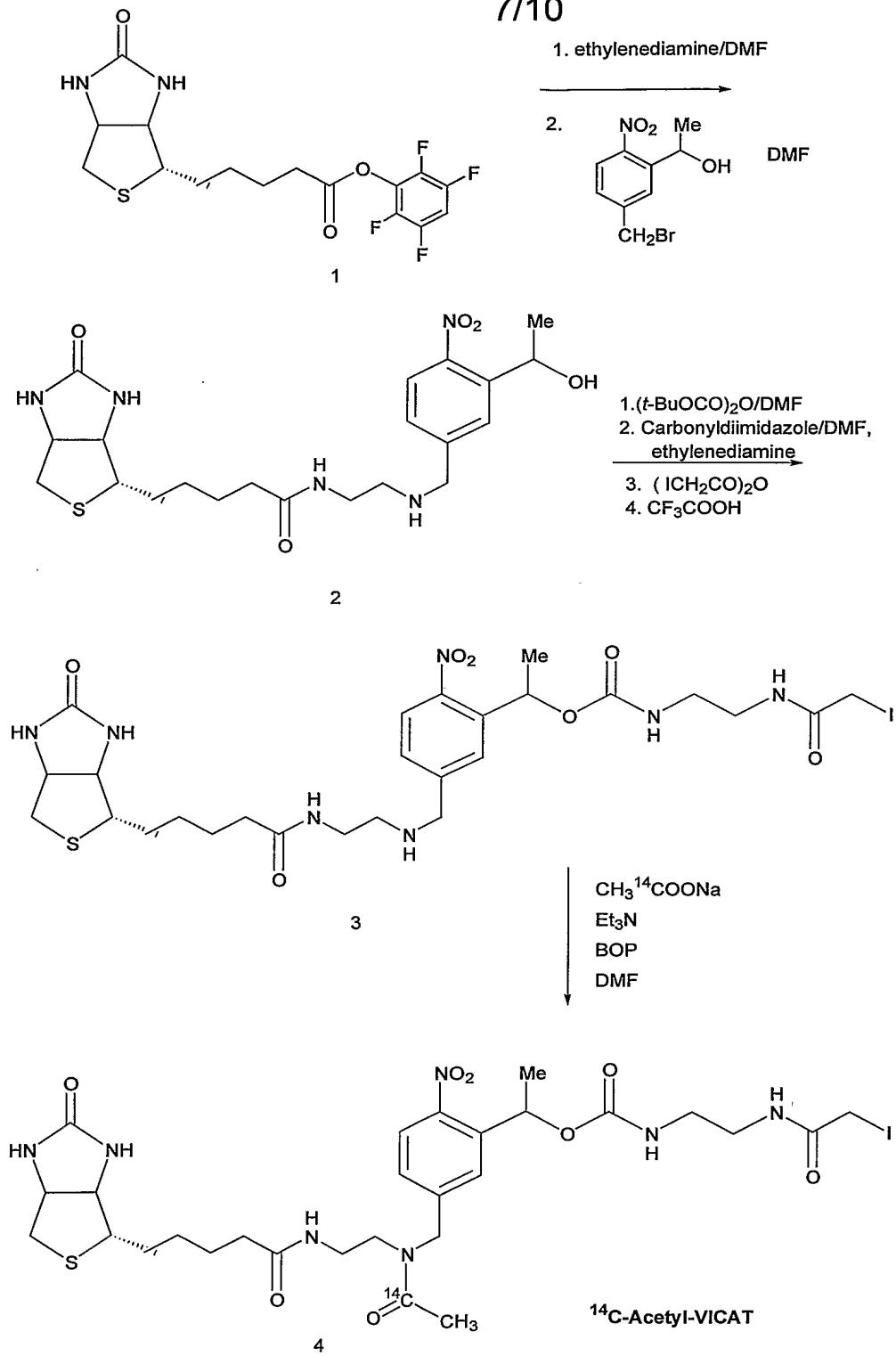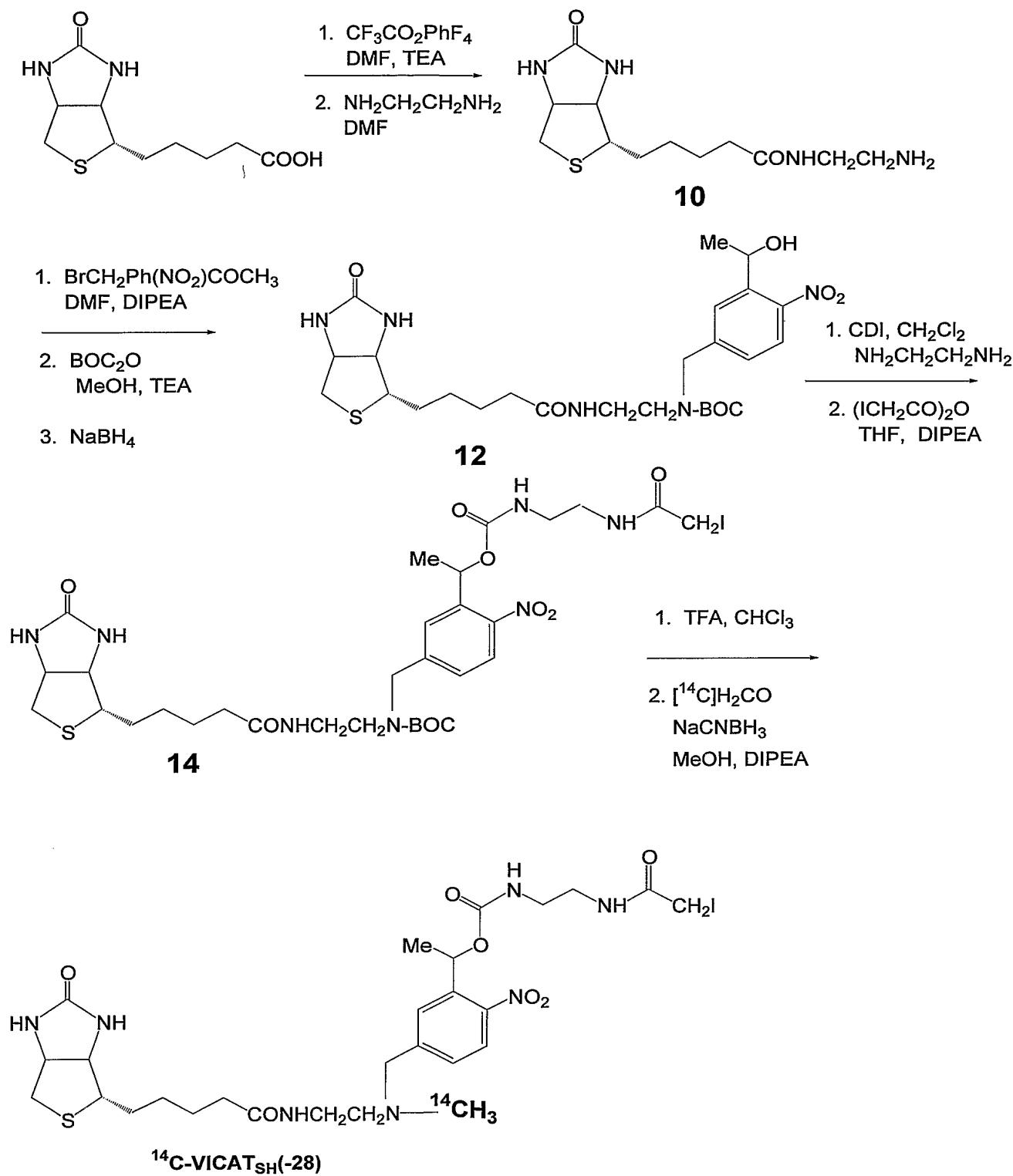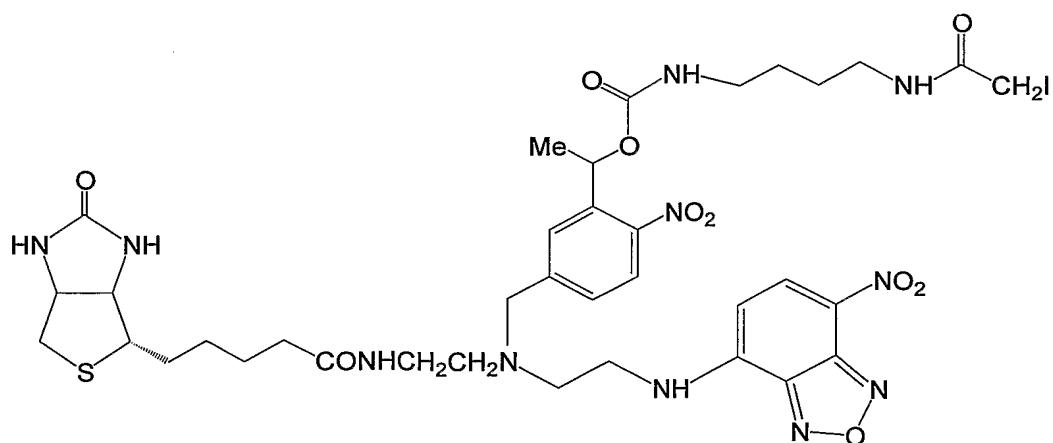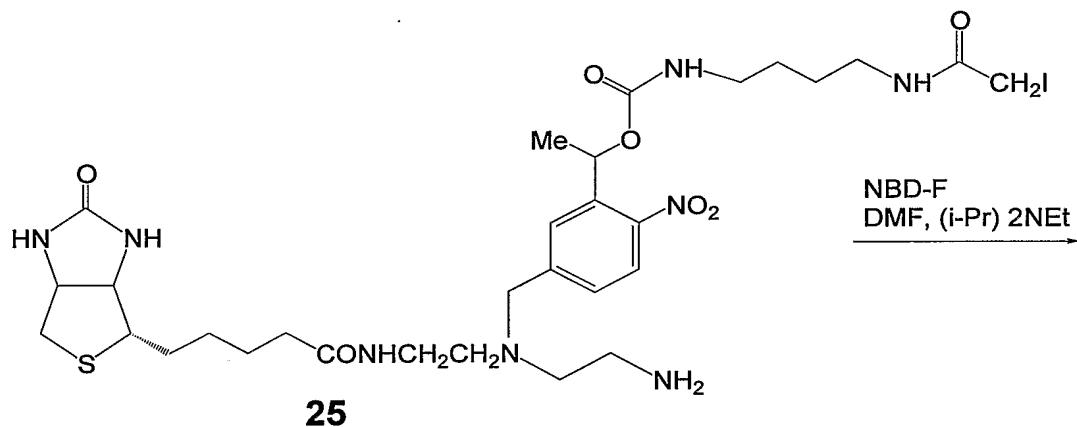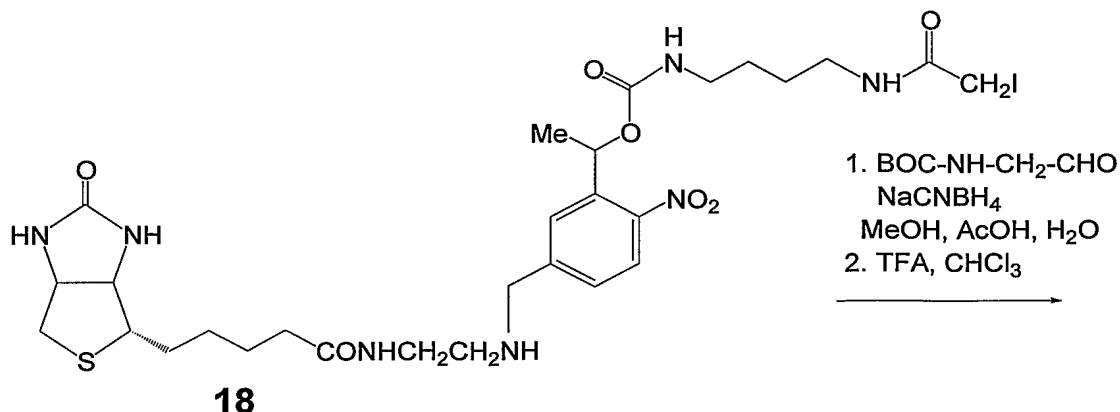
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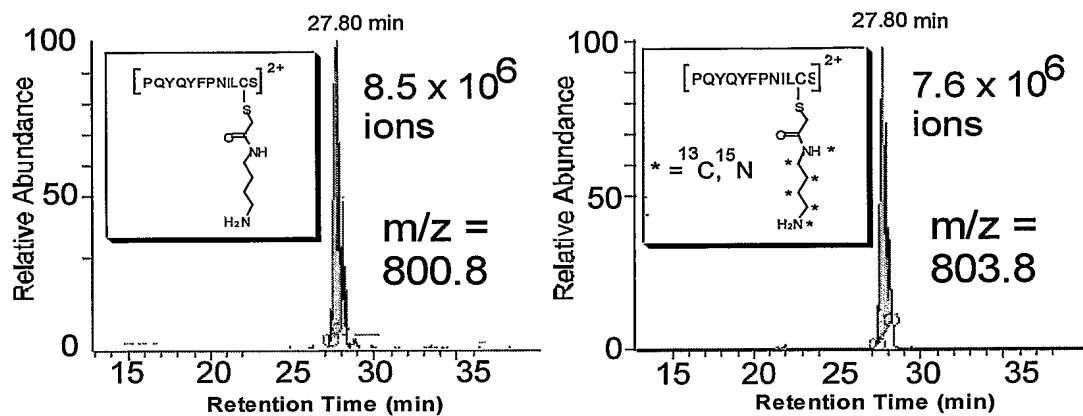
Figure 6

Figure 7

SEQUENCE LISTING

<110> THE INSTITUTE FOR SYSTEMS BIOLOGY
      UNIVERSITY OF WASHINGTON
      AEBERSOLD, Rudolf H.
      BOTTARI, Patricia Q.
      GELB, Michael H.
      TURECEK, Frantisek

<120> CHEMICAL REAGENTS AND METHODS FOR
   DETECTION AND QUANTIFICATION OF PROTEINS IN COMPLEX MIXTURES

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